Application of microsampling methods to cardiovascular medication adherence assessment

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Declaration

This thesis contains the original work of the author except where otherwise indicated.

Abstract

Cardiovascular disease remains the number one killer globally. However, adherence to medication is a major health problem. It has been reported that 50% of cardiovascular patients do not adhere to treatment. This level of nonadherence results in poor health outcomes for the patient, hospital readmissions and avoidable deaths. Wasted and unused medicines cost the National Health Service up to £4 billion annually. Commonly used methods of assessing adherence to therapy in clinical practice such as self-report, pill counting, pharmacy refill and claims data log and electronic monitors are subjective, based on proxy evidence, hence not effective. Blood drug concentrations are associated with effectiveness of the treatment and is therefore a good marker for cases of nonadherence. However, plasma and serum analysis require blood sampling by venepuncture which is highly invasive and large volumes of blood (1 – 5ml) to produce enough sample for analysis. Microsampling methods offer a cost-effective alternative to self-collect finger prick blood samples for the determination of drug levels to indicate adherence to medication.

The work undertaken in this thesis describes the development, validation and application of a microsampling based liquid chromatography – high resolution mass spectrometry (LC-HRMS) assay for simultaneous determination of eleven candidate cardiovascular drugs to indicate adherence to medication. The target drugs include amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin, and valsartan. The LC-HRMS method was validated, with results for accuracy and precision within acceptable limits; analytes were stable at room temperature in dried blood format for at least 8 weeks and hematocrit values had no significant effect. The LC-HRMS assay was used to analyse 850 dried blood samples from 141 volunteers, some of whom were prescribed one or more of the target drugs. The assay successfully identified volunteers who were known to be either adherent or nonadherent; confirmed the correct drug/drugs for multiple prescriptions; and revealed several examples of unsuspected nonadherence. These results demonstrate the possible application of microsampling based LC-HRMS assays for therapeutic drug monitoring of cardiovascular disease drugs in routine clinical practice.

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Publications and Presentations

Papers

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Posters

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Tanna, S., Bernieh, D., Alalaqi, A. and Lawson. G. Cardiovascular drug monitoring using quantitative LC-HRMS analysis of self-collected micro-volume blood samples. 15th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology 2017, Kyoto, Japan. September 2017.

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Bernieh, D. It's in a drop of blood – or is it? Research and Innovation Seminar, De Montfort University – UK, February 2017.

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Bernieh, D. Quantitative LC-TOF MS analyses of dried blood spots: Developing an assessment of medication adherence for heart disease patients. The 21st International Reid Bioanalytical Forum, Guilford, Surrey, UK, September 2015.

Contents

List of Figures

List of Tables

List of Equations

List of Abbreviations

ACE	Angiotensin converting enzyme
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism and elimination
AIDS	Acquired Immune Deficiency Syndrome
AMLO	Amlodipine
APPI	Atmospheric Pressure Photospray Ionisation
ATE	Atenolol
ATOR	Atorvastatin
β	Beta
BBC	British Broadcasting Corporation
BIS	Bisoprolol
BP	Blood pressure
CDC	Centre for Disease Control and Prevention
CID	Collision induced dissociation
CLSI	Clinical and Laboratory Standards Institute
CMS	Capillary microsampling
C_{max}	The maximum blood concentration of a drug
Conc	Concentration
CV	Coefficient of variation
CVD	Cardiovascular disease

DBS	Dried Blood Spot
DIL	Diltiazem
DOT	Directly observed therapy
DOX	Doxazosin
EBF	European Bioanalysis Forum
EDTA	Ethylenediaminetetraacetic acid
EIC	Extracted Ion chromatogram
EMA	European Medicine Agency
ESI	Electrospray Ionisation
EU	European Union
FA	Formic acid
FD	Florescence Detection
FDA	Food and Drug Administration
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GIT	Gastro intestinal tract
GP	General Practitioner
h	Hours
H_20	Water
HCL	Hydrochloride
Hct	Hematocrit

HILIC	Hydrophilic Interaction Chromatography
HIV	Human Immunodeficiency Virus
HPLC	High performance liquid chromatography
HR	High resolution
HRMS	High resolution mass spectrometry
Hz	Hertz
IS	Internal standard
IT-ToF	Ion Trap - Time of Flight
l	Litre
LC	Liquid chromatography
LC-HRMS	Liquid chromatography – high resolution mass spectrometry
LC-MS	Liquid chromatography – mass spectrometry
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LIS	Lisinopril
LLE	Liquid–liquid extraction
LOD	Limit of detection
LogD	Distribution coefficient
LogP	Partition coefficient
LOQ	Limit of quantification
LOS	Losartan
M	Molar

m/z	Mass to charge ratio
$M+H^+$	Molecular ion
$M+Na^+$	Sodium adduct ion
MA	Mass accuracy
MEMS	Medication event monitoring systems
MeOH	Methanol
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
MRM	Multiple reaction monitoring
MS	Mass spectrometer
MS/MS	Tandem mass spectrometry
MS1	Mass analyser 1
MS2	Mass analyser 2
NaOH	Sodium hydroxide
NBS	Newborn screening
NSQAP	Newborn Screening Quality Assurance Program
NDA	New drug application
ng	Nanogram
NH_4	Ammonium

NH_4OH	Ammonium hydroxide
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
PCR	Polymerase chain reaction
PET	Polyethylene terephthalate
PIL	Patient information leaflet
PK	Pharmacokinetic
pKa	Acid dissociation constant at logarithmic scale
PKU	Phenylketonuria
PO_4	Phosphate
PP	Protein precipitation
ppm	Parts per million
QC	Quality control
QqQ	Triple quadrupole
QqQ-MS	Triple quadrupole mass spectrometer
Q-ToF	Quadrupole – Time of Flight
RAM	Ramipril
RBC	Red blood cells
RE	Relative error
RMM	Relative molecular mass
R^2	Correlation coefficient

SD	Standard deviation
SFC	Supercritical fluid chromatography
SIMV	Simvastatin
SIM	Selected ion monitoring
S/N	Signal-to-noise ratio
SIR	Single Ion Recording
SLE	Solid liquid extraction
SOP	Standard operating procedure
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
$t_{1/2}$	Drug half life
TDM	Therapeutic drug monitoring
TFA	Trifluoroacetic acid
TIC	Total ion chromatogram
TK	Toxicokinetic
t_{max}	Time it takes for drug to reach highest concentration
TOF	Time of Flight
UHPLC	Ultra high-performance liquid chromatography
UK	United Kingdom
UNICEF	United Nations International Children Emergency Fund
USA	United States of America

µl	Microlitre
UV/Vis	Ultraviolet visible
V	Volts
VAL	Valsartan
VAMS	Volumetric absorptive microsampling
WHO	World Health Organization
%	Percent

Chapter 1 Cardiovascular disease and the global problem with medication adherence

Cardiovascular disease is the number one global killer, responsible for 17.7 million deaths globally every year (World Health Organisation, 2016). About a third of these deaths are needless and preventable if patients adhered to their prescription medication. The primary aim of the work presented in this thesis was to develop an evidence based method for assessing cardiovascular patient adherence to medication in routine clinical practice using microsampling methods followed by liquid chromatography high resolution mass spectrometry (LC-HRMS) analyses. An overview of the problem of nonadherence to prescribed medication, the prevalence of medication nonadherence, the consequences of nonadherence to medication and the various factors that affect medication adherence are presented in this chapter. The currently available methods of assessing patient medication adherence and their limitations are also presented and discussed.

1.1 Introduction

Cardiovascular disease (CVD) involves disorders of the heart and blood vessels, namely angina, hypertension, stroke, heart attack and heart failure (World Health Organisation, 2016). It affects an estimated 7 million people in the UK and is responsible for about 155,000 deaths each year. The economic burden of CVD is large with healthcare costs alone estimated at £11 billion every year in the UK (British Heart Foundation, 2013). An essential component of managing cardiovascular diseases properly and ensuring treatment success is to ensure patients take the prescribed medication (Keenan, 2017). Studies have demonstrated a link between medication nonadherence and adverse clinical outcomes among patients with heart disease (Ho et al., 2008; Bitton et al., 2013; Zullig et al., 2017). The drug selected and the dose prescribed should produce therapeutic drug levels in the patient's blood stream. Patient adherence to the prescription helps ensure that the blood concentration of the drug is within the therapeutic limits in order to improve treatment outcomes (Tanna

and Lawson, 2016). However, irrespective of the importance and the benefits of the treatment, appropriate use of prescribed medicine remains a big challenge for both patients and health care providers (Bosworth et al., 2011). Statistics indicate that 20% to 30% of patients do not adhere to medication regimens that are curative or relieve symptoms, 30% to 40% fail to follow regimens designed to prevent health problems and 50% of patients fail to adhere to the prescribed regimen for the treatment of chronic conditions (Pharmaceutical group of the European Union, policy statement on adherence, 2017).

A World Health Organisation (WHO) report (Sabate, 2003) states that about 50% of all patients do not adhere to their treatment regimen. Further evidence suggests that >50% of heart disease patients do not adhere to their prescription treatment (Kronish and Ye, 2013). In the UK, for example, about 370 million prescriptions were dispensed for heart diseases in 2014 and half of these were believed to be wasted because patients did not take their medicines as prescribed (British Heart Foundation, 2015). The problem of nonadherence to medication is now a global issue of striking magnitude. According to a National Institute of Clinical Excellence (NICE) guideline on medication adherence, wasted (unused) medicines cost the UK National Health Service (NHS) up to £4 billion annually (NICE, 2014; Iuga and McGuire, 2014). These statistics are shocking and the level of nonadherence to medication are startling. This level of nonadherence results in poor clinical outcomes, increased cost of care, hospital readmission, and sometimes death (La Caze et al., 2014; Yang et al., 2017).

A recent report by the British Broadcasting Corporation (BBC) health watch programme found that hospital readmissions of patients within 30 days of discharge has risen by a third (BBC, October 2017) and is estimated to cost the NHS about £1.6 billion. The cause of the rise in hospital readmissions is linked to patient nonadherence to prescription medication particularly in cardiovascular disease patients. When patients are on admission at the hospital, there are no problems with adhering to the treatment, since hospital staff (directly observed therapy) normally administer the medication. However, adherence to medication become a problem when patients are

discharged and must self-medicate at home. Nonadherence to medication is therefore a global problem affecting patients in all disease states. For example, cancer (Gupta and Bhatia, 2017; Mislang et al., 2017), diabetes (Schwartz et al., 2017), HIV/AIDS (Monroe et al., 2017), schizophrenia (Phan, 2016; Tessier et al., 2017), depression (Goldstein et al., 2017), epilepsy (Montouris and Hohler, 2016; Malek et al., 2017), asthma (Harris et al., 2016; Brandstetter et al., 2017) and malaria (Bruxvoort et at., 2014).

Hence, it is not difficult to conclude that prompt action is needed to address the problem of nonadherence to medication (Stirratt et al., 2018). Improving adherence to medication in patients taking prescription medication is pivotal to improving patient safety and the quality of healthcare tailored to the patient's needs (Du et al., 2017). It is also key to reducing unused and improperly used medications. As well as optimising the cost effectiveness of therapies and the effectiveness of managing chronic diseases. As a matter of fact, the World Health Organization has reiterated that "increasing effectiveness of adherence interventions may have a far greater impact on the health of the population than any improvement in specific medical treatments" (Sabate, 2003). This is because increased adherence to medication will lead to better health outcomes and help optimise the use of scarce health resources.

Hence as the pharmaceutical industry and clinical research develops new treatments for patients, it is equally important that measures are also put in place to identify and optimise patients' adherence to medication. Efforts are therefore required to generate innovative, objective and patient friendly means of proper assessment of medication adherence. This should be a top priority for all stakeholders' particularly health care providers and the pharmaceutical industry. Such approaches will not only improve treatment outcomes for patients and decrease health care spending, but will also increase revenue for the pharma companies. For example, Forrisier and Firlick (2014) found that an increase in medication adherence by 10 percentage points will turn into a $134 billion global pharmaceutical revenue opportunity. This represent a clear win–

win situation for all stakeholders. Hence evidence based method of assessment are required to enable proper assessment and help improve medication adherence.

1.2 What is medication adherence?

Adherence as defined by the WHO is the extent to which a person's behaviour, comprising of taking medication, following a diet or executing lifestyle changes, corresponds with agreed recommendations from a health care provider (Osterberg et al., 2005; Ferdinand et al., 2017). Most patients with chronic conditions are normally prescribed with medication that are administered orally. Thus, medication adherence is a problem for drugs administered mainly by the oral route. In this context, medication adherence refers to whether patients take their medications exactly to agreed doctor's recommendations, for example one capsule at night or two tablets a day. Tanna and Lawson (2014) argue that proper medication adherence involves six key factors. These include taking the right drug; at the right dose; at the right time; on the right schedule; under the right conditions; and with the right precautions.

1.2.1 Medication adherence - why it is important

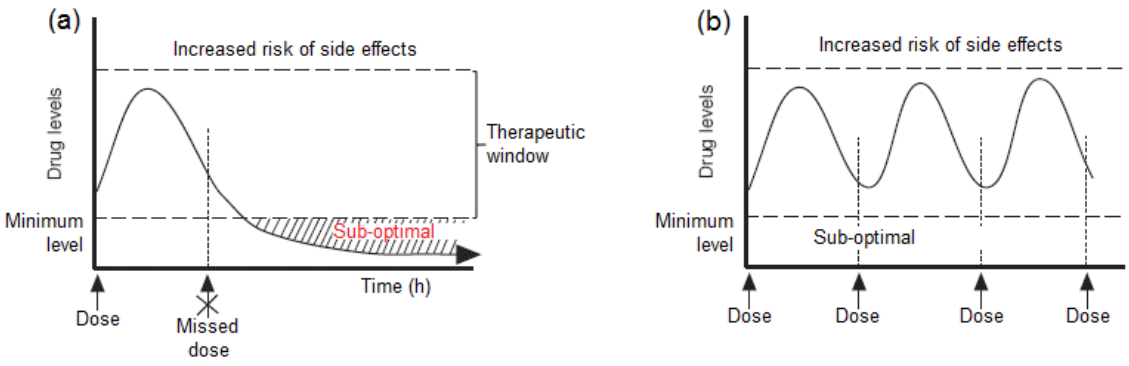


Figure 1.1 A plot of a drug concentration versus time profile in (a) nonadherence and (b) adherence

Failure to adhere to any of the identified factors affecting medication adherence could possibly lead to complications for the patient which could result in sub optimal or adverse outcomes (Otto, 2017). For example, from Figure 1.1(a), if a dose is missed, the drug concentration in the blood subsequently drops below the effective

therapeutic window with time. This leads to sub optimal levels, which has catastrophic consequence for the patient, because the treatment will have no positive effect on the clinical condition and will lead to deterioration. Whereas when the patient is adhering to the treatment, as shown in Figure 1.1(b), the blood concentration of the drug stays within the effective therapeutic window and ensures that the patient gets the full benefits of the treatment leading to an improvement in health (Roehr, 2013). More importantly, Figure 1.1(b) depicts the development of the steady-state concentration of the drug over several days, which should be maintained for any patient following a fixed dose drug regimen. This therefore shows that adherence to medication is the cornerstone for ensuring optimal clinical outcomes (Keenan, 2017). Researchers and clinicians are therefore interested in assessing adherence to medication to maximise safety and optimise treatment for patients.

There are barriers to medication adherence, health practitioners find it difficult to identify patients who do not adhere to their treatment (Wheeler et al., 2014). Policy makers, healthcare professionals and managers underestimate the chance to improve health outcomes and rationalise health expenditure by monitoring what happens after a medicine has been prescribed to a patient (Zullig et al., 2017). As demonstrated in Figure 1.1, the adherence to the prescription regimen is what ensures safe and high-quality pharmacotherapy.

Proper assessment of adherence to medication is another challenge. There is currently no gold standard measurement tool for assessing adherence to prescription medication in routine clinical practice (Lehmann et al., 2014). Current methods to assess medication adherence involves patient self-report, pill counts, pharmacy refill or claims data logs and electronic monitors (Neiheisel et al., 2014). None of these can confirm the patient ingested the medication and therefore only capture a part of the information needed for accurate assessment of medication adherence and may consequently lead to optimistic results (Tanna and Lawson, 2014). Sensors are now available that can document ingestion, but patient security and cost are of major concern (Kvedar et al., 2011; Hafezi et al., 2015).

Adherence rate is the percentage score of a patient's level of adherence to the treatment. It is normally used to express the patient's degree of adherence. However, cut-off levels, which authors use to categorise adherence to medication, is inconsistent (Nieulaat et al., 2014). It is generally accepted that rates 80% and above are adherent and rates less than 80% usually considered problematic with patients termed nonadherent (Clifford and Coyne, 2014). However, such an assumption is not always correct. The reason being that in certain disease states an adherence rate of 100% may be paramount. For example, in patients with human immuno-deficiency virus (HIV) where any reduction in adherence rate could result in the formation of drug resistant strains of the virus leading to poor clinical outcomes or at worse death (Monroe et al., 2017). Such difficulties in categorising patients' degree of adherence presents challenges for policy makers because it prevents results comparison between medication adherence studies.

Coupled with this research shows that interventions designed to increase adherence to medication have shown little improvement in adherence (Van Dalem et al., 2012; Ruppar et al., 2017). Nieuwlaat et al (2014) assessed the effects of interventions intended to improve patient adherence to prescribed medication in 182 studies and concluded that, effects were inconsistent from study to study. Sapkota et al (2014) also argues that currently available methods of improving adherence to medication for patients with chronic conditions are complex and ineffective. This may be because factors driving adherence to medication are different in different parts of the world.

More importantly, interventions designed to improve adherence cannot confirm a therapeutic level of the drug in the patients' blood, hence the full benefit of the treatment cannot be realised. For example, mHealth, which involves the use of mobile phone technology to support the delivery of medical care, has been hailed as a promising intervention. mHealth has been used by health care providers to deliver messages to increase medication adherence to CVD medication (Palmer et al., 2017). Yet there is still limited evidence for its effectiveness. Alder et al (2017) reviewed the use of mobile phone text messaging to improve medication adherence in secondary prevention of cardiovascular disease. They concluded that, there was not sufficient

evidence to support the effectiveness of text message-based interventions to increase medication adherence.

Aslani and Schneider (2014) also report a lack of consistency in terminology used to describe adherence. For example, the use of terms such as adherence and compliance. Compliance until recently was the concept used to describe patients' medication taking behaviour and was widely used in pharmaceutical and medical publications. However, it is now criticised due to the negative effect it portrays on the prescriber-patient relationship. It appears to suggest a relationship where the prescriber just decides on the best treatment option and give instructions, whilst the patient's role is to passively follow the clinician's orders. Such a connotation side-lines the patient in the treatment decision making and more importantly puts the blame of noncompliance on the patient because they were unable to follow the prescriber's instructions (Ahmed and Aslani, 2014).

Adherence on the other hand describes the extent to which a patient follows agreed recommendations by the prescriber. Adherence has been accepted mainly in the sociological and psychological literatures as a substitute to compliance with the view of emphasising that the patient has the freedom to decide whether to follow the clinician's recommendations (Barnett, 2014). Hence not following the regimen should not be reason to blame the patient. Adherence therefore develops the concept of compliance with special emphasis on the need for agreement (Ahmed and Aslani, 2014). This shift reflects the modern-day patient centred ideal of healthcare where clinicians and patients agree on the plan of treatment, rather than patients just following the prescriber's instructions.

Another terminology which has recently being introduced in the UK is concordance. It refers to the bigger concept of patient support in medicine taking and focuses more on the patient-prescriber relationship. It stresses on the need for the prescription to represent a shared decision. Concordance, takes into full consideration the beliefs and preferences of both the prescriber and the patient with the recognition that the patient's views are supreme. The foundation of concordance includes the level of

information given to patients, explanation of side effects, the cost of medication and the effect on lifestyle. It is worth mentioning that the idea of concordance is related to but not the same as adherence (Horne et al., 2014; Khair, 2014).

1.3 Medication nonadherence

Nonadherence to medication refers to when patients fail to follow agreed recommendations by their doctor with regards to their prescription treatment. It remains a major problem, especially for people with chronic diseases (Bitton et al., 2013; Palmer et al., 2017). For example, medication nonadherence is known to raise the risk of revascularization, heart disease related hospitalization and death in cardiovascular patients (Ho et al., 2008). Medication nonadherence as explained by the WHO can take any of the following forms, failing to initially fill a prescription, failing to refill a prescription as advised, skipping a dose or doses, administering more of a medicine than prescribed, taking a dose at the wrong time, and prematurely stopping medication.

The National Institute for Health and Care Excellence (NICE) classifies medication nonadherence into two overlapping categories, these are intentional and unintentional (NICE guidelines, 2009). Intentional medication nonadherence refers to when the patient decides not to follow the treatment recommendations due to beliefs and perceptions about the treatment, example deliberately skipping a dose to avoid side effects or due to cost of the medication. It's been reported that about half of medication nonadherence cases are intentional (Pound et al., 2005; Mukhtar et al., 2014).

With unintentional, the patient wants to follow the treatment recommendations but has practical problems. Examples include poor recall, difficulty in understanding the instructions or inability to pay for the prescription. It is normally characterised by several behavioural patterns. These include failure to collect prescriptions, not following instructions with regards to doses. In both situations, the outcome may be dangerous. NICE however are of the view that the patient should not be blamed and

advocate a non-judgmental approach in which the patient's perceptions and preferences can be explored (NICE guidelines, 2009).

1.3.1 Consequence of nonadherence to medication

The consequences of non-adherence to prescribed medication include poor health outcomes for patients, missed opportunities for therapeutic gain, and increased cost of health care mainly linked with deterioration of the medical condition being treated, increased referrals, additional laboratory investigations and death. (Sabate, 2003; Mahoney et al., 2008; Boswell et al., 2012; Chisholm-Burns et al., 2012; Clifford and Coyne, 2014). The effects of nonadherence to medication affects not only the patient, but the health care provider and the pharmaceutical industry as well. Hence the impact of nonadherence can be grouped into consequences for the patient, pharmaceutical industry and health care provider. This confirms why the problem of nonadherence to medication should be a top priority for all stakeholders. Below is a summary table of the consequences of nonadherence to medication to the three identified groups.

Table 1.1 Consequences of non-adherence to medication

Group	Consequences
Patient	• Suboptimal clinical outcome for patients (Piepoli et al., 2016). • Missed opportunities for therapeutic gain (McCarthy, 1998). • Adverse effects on the health and wellbeing of patient. Nonadherence to immunosuppressive drugs has been linked to heart, kidney, and liver transplant failures (Rapoff, 2010). • Reduce cost effectiveness of the treatment. • Disease related complications resulting in mortality. • Negatively impact medical decisions by health professionals. • Request for unneeded laboratory test, dose adjustment or unnecessary change in prescription.
Pharmaceutical industry	• Loss of revenue due to medication wastage, classified as either as therapeutic loss or material waste (IMS Health, 2013; Hazel and Robson, 2015). • Cost to global pharmaceutical market is estimated at $637 billion (Forrisier and Firlick, 2014).
Healthcare provider	• Increased cost of care, unnecessary clinical appointments and emergency room visits (Barnett et al., 2014). • Hospital re-admissions, hundreds of billions of dollars spent on re-admissions annually (Boswell et al., 2012). • Significant human cost estimated at 195,000 deaths in Europe (Pharmaceutical Group of the European Union, 2008) and 125,000 deaths in the US (Boehme et al., 2017; Ferdinand et al., 2017). • Wasted resources due to non-use of medicines funded by healthcare systems (Bitton et al., 2013). • Drug resistance, e.g. Resurgence of infectious diseases such as tuberculosis, development of drug resistant forms of the HIV-AIDS virus resulting in high viral loads (Chisholm-Burns et al., 2011). • Reduction in life expectancy of the aging population (Boehme et al., 2017).

1.3.2 Prevalence of medication nonadherence

High rates of nonadherence exist in several disease states. Examples include cardiovascular diseases, cancer, diabetes, asthma, mental health (neurological disorders) (Oung et al., 2017). Medication adherence assessment suggest an average nonadherence rate of 50% with huge variation in patient populations based on disease condition (DiMatteo, 2004). For example, higher rates of nonadherence have been reported in psychiatric disorder patients' example elderly patients with depression or cognitive disorder (Velligan et al., 2009). As well as in patients undergoing treatment for multiple conditions e.g. hypertensive patients with diabetes (Ho et al., 2009, Brown et al., 2011, Monroe et al., 2017). Even when experiencing symptoms, more than 50% of asthmatic patients could not achieve the desired level of inhaled corticosteroid use (Adherium, 2017). Medication nonadherence is also prevalent in the treatment of malaria and HIV - AIDS, which is common in sub Saharan Africa and Asia (Taneja at al., 2013).

Taking Europe for example the rate of nonadherence to medication varies between 43% and 60%, whilst in the US it is estimated to be around 50%. However, such information does not take into consideration the extent of variation that may occur in certain disease states or patient groups (Larsen et al., 2009). For example, in cancer patients, published figures reveal wide variations in nonadherence rates. From as high as 73 – 86% for haematological cancers compared to 12 – 47% in breast cancer patients and about 3% for patients with ovarian cancer. (Partridge et al., 2002; D'Amoto, 2008; Ruddy et al., 2009; Mathes et al., 2014 and Mitchell et al., 2014).

In the USA, more than 25% of patients released from hospital following acute myocardial infarction do not collect their medicines within 7 days of discharge. In addition, about 34% of patients stop taking at least one of their medications, with 12% stopping all medications within a month of being discharged from hospital. In a study by Jackevicius et al (2008) looking at medication usage consistency over a period of 6 to 12 months, 56% of heart patients were found to be nonadherent to statins and 54% were nonadherent to beta blockers (Ho et al., 2009). In a study by Kronish et al (2011),

nonadherence rates were as high as 72% for β-blockers and 35% for angiotensin 2 receptor blockers. In a study conducted in China, nonadherence rates to antihypertensive medication was found to be 35% among patients. (Lee et al., 2013).

In HIV patients, antiretroviral therapy is required to reduce viral load in the blood. Hence living a healthy life depends on adherence to these medications and to prevent drug resistance. However, treatment regimen complexity makes it difficult for patients to adhere to therapy. Nonadherence rates of 5 – 30% have been reported in HIV patients in the US, meanwhile rates below 5% is required to achieve consistent viral suppression. Naderi et al (2012) also reported variation in nonadherence based on whether medications are prescribed for primary or secondary prevention, with high rates of nonadherence seen in primary prevention. There has also been documented evidence of poor adherence in clinical trials participants, where patients have been carefully chosen. Despite the differences in the methods used to measure nonadherence in these cited studies, it is obvious that prevalence of nonadherence to medication is common among patients with chronic conditions.

1.3.3 Factors affecting medication adherence

Factors driving adherence to medication are different in different parts of the world. Taking Europe and Africa for example, the system of healthcare in these continents are totally different. A patient in Europe may book an appointment to see the doctor and afterwards pick the prescription medication from the pharmacy at a subsidized cost paid through insurance contributions by the patient. Whilst in Africa, no insurance contributions are made by patients towards healthcare, hence patients may pay to see a clinician and fully pay for the prescription. Hence cost of treatment (Figure 1.2) may be a driving factor in Africa, but not in Europe. In a study by Awad et al (2017), assessing medication adherence among cardiac patients in Sudan, high cost of medication, polypharmacy and poor clinician/patient communication about the treatment were identified as the main barriers to adherence. Abdulazeez et al (2014) also identified a direct link between patient financial status and adherence among patients taking prescribed diabetic medication in Nigeria. In view of this, interventions

designed to increase adherence in Europe may not necessarily apply in Africa since the underlying factors may be different and the awareness of this by stakeholders is very important.

Nobre and Domingues (2017) reported many factors affecting patient medication adherence. To increase patient adherence to medication, there is the need to understand the reasons why nonadherence occurs. These reasons are seen to be multifactorial (Monroe et al., 2017) and can generally be grouped into patient related, clinician related and health system related factors; the WHO further classifies these factors into five subclasses (Figure 1.2): socioeconomic, healthcare system, disease related, therapy related and patient related factors (Iuga and McGuire, 2014; Ferdinand et al., 2017).

1.3.3.1 Patient-related factors

These factors are further classified into demographic (gender, age, marital status and level of education), social and psychological factors (patient's beliefs, attitude and motivation towards the treatment). They are mainly due to a lack of understanding of the disease condition, lack of patient involvement in the treatment decision making and medical illiteracy (Halladay et al., 2016; Khalesi et al., 2017). In the US for example, over 80 million adults are reported to have inadequate health literacy increasing their risk of hospital readmissions and suboptimal clinical outcomes (Mayo-Gamble and Mouton, 2017). Other causes include the health beliefs of the patient and attitudes with regards to the treatment effectiveness, past treatment experiences, religious and cultural beliefs about the condition, mental health problems and an utter lack of motivation (Figure 1.2). In countries where patients pay for treatment, high prescription cost and longer pharmacy waiting times has been identified as barriers to medication adherence (Otto, 2017). In patients with mental illness a lack of social or family support also serves as a predictor of nonadherence (Baroletti and Dell'orfano, 2010; Brown and Bussell, 2011). In a study by Chen et al (2014), gender was found to be specifically associated with adherence to antihypertensive medication and awareness of the factor was key to delivering best interventions.

1.3.3.2 Clinician-related factors

Clinicians normally fail to identify patients who are nonadherent and sometimes worsen the situation by the prescribing of complex medication regimen especially in patients with co-morbid conditions (Monroe et al., 2017). Kronish et al (2013) reported that the most common approach to assessing medication adherence by clinicians in clinical practice is clinical judgement. However, there is evidence that prescribers are poor at judging the extent to which their patients take their medication (Zeller et al., 2008). Hence prescribers underestimate the problem of nonadherence to medication which were attributable to many factors including the lack of awareness of the high prevalence of nonadherence, not believing that their own patients will not adhere to their regimen, lastly relying heavily on patient self-report whilst patients over estimate their level of adherence (Singh, 2017).

Some healthcare providers also fail to give full explanation about the benefits and side effects of the treatment (Figure 1.2). Improving patient adherence, Martinez and Finken, (2006) showed that patients who are satisfied with their relationship with their clinicians were adherent to their diabetes treatment, whilst patients who rated the relationship with clinician as poor were nonadherent to oral medication. In countries where patients pay for the service, proper considerations are normally not given to the financial constraint to the patient, taking into consideration the duration of the treatment. A poor clinician communication skill also complicates the patient's understanding of his or her condition, any likely complications and the essence of adhering to the treatment (Schoenthaler et al., 2017). Finally, lack of proper communication between healthcare personnel involved in the patient care could sometimes lead to medication errors and hence avoidable readmissions (Ferdinand et al., 2017).

1.3.3.3 Health system factors

Fragile healthcare systems facilitate nonadherence to medication by limiting the coordination of care for the patient. Longer waiting times at the clinic or pharmacy have been identified as a barrier to patients' medication adherence (Ferdinand et al.,

2017). Lack of support from healthcare team members and poor clinician-patient relationship have been identified as determinants of medication nonadherence. In addition, health systems that lacks the ability to provide education on treatment or follow up promote nonadherence to treatment. E.g. having patient information leaflets written at a high level of literacy may make it difficult for patients to understand (Schoenthaler et al., 2017).

It is worth noting that several interconnections exist between all these factors and demonstrate clearly that medication nonadherence is indeed a complex problem (Kardas et al., 2013). Mathes et al (2014a) concluded in a review that just a small number of factors regularly influenced adherence. In addition to these factors unemployment, belonging to an ethnic minority and cost to the patient (Figure 1.2) demonstrated a negative influence on medication adherence (Dhaliwal et al., 2017). Hence indicating the involvement of social factors. It is therefore not surprising that guidelines to improve adherence have been issued worldwide given the magnitude of complexity (Mathes et al., 2014b). In a review by Ruppar et al (2015) they identified 23 international and national clinical practice guidelines intended to assist healthcare providers. These guidelines were from the USA, UK, Canada, Spain and Australia.

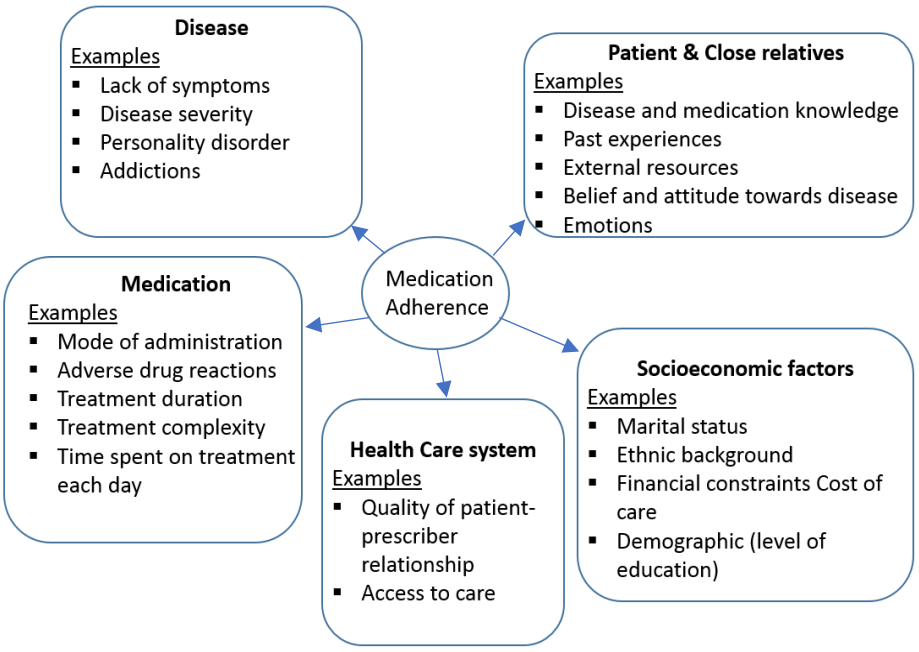


Figure 1.2 A subdivision of factors affecting medication adherence

1.4 Methods of assessing adherence to medication

Assessing the problem of nonadherence to medication is further challenged by the range of tools that are available for assessment. Ahmed and Aslani (2014) call for an implementation of robust systems within primary healthcare which will enable routine assessment as well as monitor patients' adherence to medication. They believe this will aid in identifying nonadherence to medication and allow health providers to address the problem to allow optimisation of treatment. Several methods have been studied to measure adherence to medication, but there is currently no universally accepted gold standard measurement tool for assessing adherence to therapy in clinical practice (Lehmann et al., 2014). A range of methods have reportedly been used to assess adherence to medication, each having its own advantages and disadvantages. The mode of assessment can be grouped into indirect and direct methods.

1.4.1 Indirect methods of assessing medication adherence

Indirect methods are subjective and assume ingestion based on proxy evidence and normally do not prove the presence of the medication in the body. Examples include self-report by patients, electronic monitors, pharmacy refills and claims data, and pill counting (Lawrence et al., 2017). Recent developments include computerized logbooks and the use of downloadable apps on smart phones for real time assessment and alerts (Marawski et al., 2017). Indirect methods therefore capture just a part of the information needed for proper assessment. For example, pill counting provides no information on dose timing which may be crucial to determine outcomes clinically.

1.4.1.1 Self-report

Self-reporting by the patient or their carer is the simplest method of assessing adherence. It is a way of asking the patient for their subjective rating of medication adherence. It is done through interview, questionnaire or using a diary. It is cheap and easy for the patient to perform but often inaccurate because it does not provide a precise measure of adherence (Pérez-Escamilla et al., 2015). Whether a patient tells the truth may depend on the clinician/patient relationship and the way the questions

are asked (Neiheisel et al., 2014). Also, patients may be tempted to exaggerate their adherence if they believe that reports of nonadherence will disappoint their clinician (Gupta et al., 2016). They may also overestimate adherence through forgetfulness (Zullig et al., 2017). Thus, doctors may prescribe alternative medication, request additional laboratory tests, or arrange specialist consultations to evaluate the cause of unexplained or persistent symptoms which may have been avoidable (Wagner and Rabkin, 2010).

1.4.1.2 Pill counts

Pill counts basically involve counting the dosage units that the patient has not taken by the scheduled appointment or clinic visit (Haynes et al., 2016). The returned dosage units are counted and compared with the number of units received by the patient in the most recent prescription and the length of time since the medication was dispensed. Adherence is then calculated by subtracting the number of unit returned from the number of units issued. This gives an indication of the number of medication used by the patient within a specific period (Neiheisel et al., 2014). However, the accuracy of pill counts in estimating medication adherence is questionable because some patients may intentionally not return their medications (Lawrence et al., 2017).

1.4.1.3 Electronic monitors

Electronic monitors such as medication event monitoring systems (MEMS) record when a prescription bottle is opened, pills are removed from a dosage pack, or when an inhaler is activated. They generate data which provides detailed profile of medication usage over time (Park et al., 2015). However, Zullig et al (2017) argues that the opening of the container does not guarantee ingestion of the medication. The dose could be discarded by the patient. Furthermore, electronic monitors are costly and each device can monitor only one medication. Another disadvantage is that they cannot be fitted to many of the dosage forms and packaging used in routine care. Hence the focus should be centred on developing and improving methods of assessing if the medication was consumed (Seabury et al., 2014).

1.4.1.4 Pharmacy refill and claims data

Pharmacy databases can be used to check when prescriptions are initially filled, refilled or prematurely discontinued. This involves electronic review of prescription claims in databases containing patient's records (Gupta et al., 2016). Some patients may use more than one pharmacy, hence access to all pharmacies patronised by the patient is necessary for accurate assessment (Lawrence et al., 2017). However, such reviews provide no confirmation that the medication was ingested by the patient and have a high tendency of yielding optimistic results.

1.4.2 Direct methods of assessing medication adherence

Direct methods are objective, examples include directly observed therapy (DOT) and biochemical measurements that detect the presence of medication in the body. For example, analysis of bio fluids for the presence of the drug or its metabolite or detection of a biomarker that is given with the drug. Such test can be done at specified intervals or randomly when it is appropriate. It provides evidence that the patient has taken the medicine (Morrison et al., 2015). Hence direct methods of assessment are the most accurate means to measure adherence (Gupta et al., 2016; Aonuma et al., 2017). The down side to these approaches are cost in relation to patient and clinician time. Sampling may also require a visit to the clinic. Recent developments include the testing of ingestible sensors which are incorporated into pills as a means of documenting ingestion (digital pills) (Gupta et al., 2016). However patient security and cost may be of concern when sensors are used (Kvedar et al., 2011; Chai et al., 2015; Hooman et al., 2015). The effect of cost linked with direct approaches can be significantly reduced with no disadvantage to the information generated by using a finger prick blood sample collected as a dried microsample for the quantification of drug or biomarker level to indicate adherence (Tanna and Lawson, 2016).

1.4.2.1 Directly observed therapy

Directly observed therapy (DOT) involves invitation of patients to the hospital to take part in ingestion of their mediations under the direct supervision of a nurse. Hameed et al (2015) reported in a study involving the use of DOT for assessing antihypertensive medication adherence, that 25% of nonadherent patients developed symptomatic hypotension. Gupta et al (2016) also states that there are reports of nonadherent patients being admitted in hospital due to sudden drop in blood pressure triggered by the sudden ingestion of previously avoided prescribed medication. The main limitations of DOT include costs and labour. Patients may have to travel long distances to get to the hospital and may spend half of the day at the clinic. In addition, supervision of patients may have to done by trained personnel.

1.4.2.2 Drug assay in biofluids (Biochemical measurements)

This mode of assessment involves the assay of biological fluids example blood, urine, saliva and sweat for the presence of the drug or its metabolite. It may also involve the addition of non-toxic biological markers to medications and assessing their presence in biofluids such as blood, plasma, serum or urine (Singh, 2017). It provides confirmation that the patient has received the dose. However, the technique required for sample collection of biofluid such as blood may be invasive. In addition, variation in individuals' metabolism and volume of distribution may affect drug levels. In the case of urine, the presence of a metabolite may not give a true indication of the concentration of the drug in blood. In addition, the effect of collection times for urine sample on results have been shown to produce a white coat adherence effect. Where there is marked improvement in adherence to medication in just a few days to a scheduled clinical appointment (MacLaughlin et al., 2005). Urine analysis has been used to investigate the presence of prescribed CVD drugs for patients exhibiting 'resistant hypertension' (Tomaszewski et al., 2014; Hamdidouche et al., 2015; Lawson et al., 2016; De Nicole et al., 2017, Hamdidouche et al., 2017) but this approach provides no information of the drug levels in the patient's blood. Other studies (Olds et al., 2015; Moore, 2015; Ferrari et al., 2017) have investigated the use of saliva and hair

in adherence to medication research due to the benefits they offer over blood and urine such as a non-invasive, painless and stress-free sampling, wide detection window and ability to provide a measure of long term drug use for hair samples, low cost and no requirement to visit a clinic or hospital for the sample to be taken.

1.4.2.3 Dried blood spot (DBS) microsampling

A significant limitation in assessing adherence to medication using a direct method involving analysis of biological fluid such as blood is the need for blood sampling. Conventional plasma or serum based drug quantification methods often require large volumes of blood (1 – 5ml) at each sampling time to produce enough plasma/serum for analysis (De Nicolo et al., 2016). There is the need for patients to visit a phlebotomist (specialist collection). The process of blood sampling is also highly invasive and makes patients uncomfortable (De Nicolo et al., 2017). This poses practical challenges in performing medication adherence studies to allow treatment to be optimised for patients. Dried blood spot represents an alternative matrix for measuring blood drug concentrations. Requiring only a micro blood volume (<30µl) it has great potential in overcoming the barriers associated with blood collection using venepuncture.

The use of DBS obtained from heel or finger prick, and spotted onto filter paper for collection and analysis of human blood dates back to the early 1960's when Dr. Robert Guthrie used the technique to measure phenylalanine in newborns for the detection of phenylketonuria (Shah et al., 2013). By using DBS sampling, public health laboratories screened more than 95% of all newborns in the USA for inborn metabolic disorders (Deep et al., 2012). Over the past several years dried blood spot (DBS) sampling has emerged as a pertinent method in both qualitative and quantitative bioanalysis. Advantages of DBS such as low blood volume requirement, transportation and storage without special treatment, better analyte stability, enhanced clinical cooperation in clinical trials and reduced unforeseeable exposure of biohazard to analysts make it feasible for blood sampling (Sharma et al., 2014). Hence, the ease of use and low cost (<£2.00) of the DBS sampling platform compared to blood sampling by venepuncture

makes it ideal for assessing adherence to medication. In addition, DBS sampling enables samples to be collected by patients themselves or parents/guardians at home. Samples can then be posted by regular mail to the laboratory. This allows for convenient monitoring at any desired sampling time and for the results to be readily available at the clinic during a routine check-up (Spooner, 2013).

For example, Martial et al (2016) performed a rigorous cost analysis on the potential savings achieved by using DBS sampling compared to conventional blood sampling at the clinic for TDM in renal transplant and hemato-oncology pediatric patients. Their findings revealed that, using DBS samples collected at home could lead to a cost reduction of 61% for renal transplant patients and 43% for hemato-oncology patients. The key factors contributing to the huge difference between DBS "at home" sampling and conventional sampling at the clinic were additional costs of travel, lost productivity for the caregiver and the nurse's time spent collecting samples.

Dried blood spot sampling has been used in the quantification of antiepileptic drugs (Shah et al., 2013a; Shah et al., 2013b; Linder et al., 2016; Das et al., 2017), immunosuppressants (Koop et al., 2013; Li et al., 2010; Koster et al., 2017; Martial et al., 2017; Veenhof et al., 2017), antiretroviral drugs for HIV - AIDS (Castillo-Mancilla et al., 2013; Hoffman et al., 2013; Zheng et al., 2014; Alcaide et al., 2017), cardiovascular therapy drugs (Lawson et al., 2012; Lawson et al., 2013; Tanna et al., 2014; Bernieh et al., 2017), antibiotics (Al-Ghazawi et al., 2010; La Marca et al., 2012; Hawwa et al., 2014; Vu et al., 2014; Barco et al., 2017; Weber et al., 2017), antidiabetics (Aburuz et al., 2006) and antimalarial (Blessborn et al., 2010). This demonstrates that DBS microsampling is a viable option compared to tradition plasma or serum sampling technique.

1.4.3 Strength and limitations of the various methods of assessing medication adherence

Each of the available direct and indirect methods used for assessing adherence discussed in Section 1.3 has certain flaws which limit their accuracy, reliability or practical application. Most of the available methods function as indicators of

adherence rather than exact, quantitative measure of adherence (Singh, 2017). There are also wide variations in the rates of adherence stated in most of the studies and this is because each method captures different information about patient's medication taking behaviour. Table 1.2 summarises the strength and limitations of various methods of assessing medication adherence.

Table 1.2 Strengths and limitations of the various methods of assessing adherence to medication

Strength	Self-Report	Electronic monitors	Pharmacy Database	Pill Count	DOT	Urine drug levels	Plasma drug levels	Drug Biomarker Assessment	Drug monitoring using DBS
Easy to administer	√	√		√	√				√
Cheap/ global	√			√					√
Non- Invasive	√	√	√	√	√	√			√
Data on dates and time of use		√			√		√	√	√
Confirms medication usage					√	√	√	√	√
Provides quantitative information							√	√	√
Provides PK data						√	√	√	√
Limitations									
Invasive							√	√	
Expensive equipment		√				√	√	√	√
No confirmation of adherence	√	√	√	√		√			
Limited to certain dosage forms		√							
References									
	Wagner & Rabkin, 2000	Park et al., 2015	Lawrence et al., 2017	Neiheisel et al., 2014	Hameed et al., 2016	De Nicole et al., 2017	De Nicole et al., 2016	Singh, 2017	Lawson et al., 2012

1.5 Gaps in the literature

Irrespective of the volume of work done on assessing patients' adherence to medication, there are gaps in knowledge concerning how patient adherence to medication can be objectively assessed (Gupta et al., 2016) and how developed adherence interventions can be tailored to suit patients (Seabury et al., 2014). Interventions offered and tested to increase adherence to medication though many, are complex and mostly ineffective hence the full benefits of treatment are not achieved (Nieuwlaat et al., 2014). Examples of such interventions are: behavioural counselling (Roberts et al., 2014), interventions utilizing pictorial aids of medication (Monroe et al., 2017), interventions utilising smartphone apps (mHealth) that provide reminders to take medication, education, and enable social interactions between individuals with similar health concerns (Morawski et al., 2017), interventions facilitating patient provider communication, providing patient education and psychosocial support (Zullig et al., 2017). Yet despite these interventions the rate of nonadherence to CVD medication is still high and carries a significant human cost (Granger et al., 2015).

There is also no universally accepted assessment tool for inferring adherence in clinical practice. Hence the wide variety of adherence measures limits comparison between studies (Nieuwlaat et al., 2014). Most of the assessment tools available are indirect methods which are not effective because they are based on proxy evidence and therefore yield optimistic results. Most importantly, these adherence measures are not able to establish any direct link between improved medication adherence and clinical outcomes which is essential if the patient is to gain the full benefit of the treatment. This is because they provide no information on the therapeutic levels of the medication.

Cardiovascular disease affects mainly the elderly, thus as the global population of the elderly increases and patients are prescribed more medicines (polypharmacy – not less than five medicines) factors such as individual variation in drug metabolism and possible drug-drug interactions become more important (Gonzalez et al., 2015). Hence

monitoring therapeutic drug levels by direct analyses of patient blood samples can offer clinicians very valuable information about possible drug-drug interactions, side effects occurring from the co-administration of several cardiovascular drugs and a patient's adherence to a complex prescribed medication regimen by looking at the data from a patient's blood drug concentrations for all prescribed medicines. Blood drug concentrations are associated with effectiveness of the treatment and can therefore be a good marker for cases of nonadherence. For example, in patients exhibiting resistant hypertension, application of therapeutic drug monitoring (TDM) of CVD drugs in plasma and serum samples to recognize poor adherence is known to prevent some unnecessary invasive techniques employed in its management example (renal denervation or baroreceptor stimulation) (Brinker et al., 2014; De Nicole et al 2016).

Thus, simple and objective adherence measures are therefore required to effectively assess patient adherence to medication. Such approaches will provide concrete scientific evidence upon which medical professionals can make sound clinical decisions. Direct method of assessment is seen as the best way to measure medication adherence to cardiovascular medication since it confirms the presence of the medication in the body. Direct measurement of therapeutic drug levels post-dose is known to be of significant value as a means of dose optimisation for individual patients, particularly with cardiovascular disease since most CVD drugs have high inter-patient variability (Aonuma et al., 2017). This is because it makes the treatment effective and safe for the patient by ensuring the patient blood drug concentration is within the safe and effective therapeutic window.

However, assessment by direct methods has its own challenges. Therapeutic drug levels are conventionally monitored using either whole blood or plasma samples. The reason is to ensure that the sample accurately represents the analyte in circulating blood (Verhaeghe et al., 2017). Hence data obtained from the routine analysis of plasma or serum can confirm satisfactory adherence to medication by confirming a therapeutic level of the drug in the patient's blood (De Nicolo et al., 2016; Tanna and Lawson, 2016). However, to generate the required plasma, about 5ml liquid blood

sample will be needed from the patient at each sampling time which will require an invasive blood sampling procedure (venepuncture) and the service of a phlebotomist. This makes such an approach not feasible for routine clinical testing.

Urine samples has been used in adherence studies, but can only confirm that drugs were ingested based on the detection of either the drug or its metabolite (Tomaszewski et al., 2014; Hamdidouche et al., 2015; Lawson et al., 2016; De Nicolo et al., 2017; Hamdidouche et al., 2017). However, this approach provides no information on the drug levels in the patient's blood, which is key to relate adherence to positive clinical outcomes (Morrison et al., 2015; Tanna and Lawson, 2016, Bernieh et al., 2017).

The study aims to address these limitations by developing an evidence based bioanalytical method for inferring patient's adherence to commonly prescribed cardiovascular medication using dried blood microsampling methods coupled with liquid chromatography – high resolution mass spectrometry (LC-HRMS) analyses. Blood microsampling is a simple sampling method which is minimally invasive and offers better patient comfort compared to venepuncture.

Blood microsampling based CVD drug concentration measure will allow the inference of medication adherence by confirming a therapeutic level of the drug in the patients' blood. Which is important to relate adherence to positive clinical outcome. The information derived from such an approach will help clinicians in the clinical decision making. It will enable the implementation of routine TDM of cardiovascular medications in everyday clinical practice. Data from microsampling based CVD drug concentration measurements could be used to adjust doses for individual patients. Hence enabling the patient to achieve blood drug concentration within the therapeutic range at which patients are expected to exhibit an optimal clinical response (Milosheska et al., 2015). Thus, allowing the full benefit of the treatment to be realised.

To date, only a few bioanalytical assays are reported in literature for the quantification of CVD drugs using microsampling methods (Lawson et al., 2012; Lawson et al., 2013;

Tanna et al., 2015) and none of them can quantify a wide panel (≥10) CVD drugs in the same run. Most importantly, the limit of quantification for the target analyte reported in these papers are poor, hence quantification of CVD drugs in volunteer samples at very low concentrations may yield negative results. Since heart disease patients are normally treated with combined therapies (Gonzalez et al., 2015), the adoption of multi panel drug assays is practically mandatory.

The novelty of this research resides in the ability to simultaneously quantify a wide panel of commonly used cardiovascular drugs: amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan in microvolume blood samples collected using microsampling methods coupled with LC-HRMS analyses for the inference of cardiovascular medication adherence. LC-MS/MS remains the gold standard in regulated bioanalysis, but the advantage of high-resolution full scan mass spectrometry analyses for TDM of cardiovascular drugs is that all the mass spectral data from the sample is collected. This allows the data to be revisited at a later time if it becomes clinically important to the management of the patient's condition. For example, in situations where a patient may be taking other medications in addition to the prescribed drug which the clinician is not aware of. Possibly causing drug interactions, which could lead to potentiation or inhibition of one drug response over the other.

Moreover, this assay stands out for its sampling simplicity using microsampling methods, which offer the possibility for self or "at home sampling" eliminating the requirement for clinic visits and the services of a phlebotomist. The assay is also unique because of its potentially wide applicability to other conditions with known prevalence of medication nonadherence such as diabetes, depression and cancer. In addition, the use of microsampling methods enables the wide applicability of the assay in resource-limited areas of the world where there are no logistics for transportation and storage of liquid blood matrix.

The objectives of this thesis are:

- To develop and validate microsampling based LC - HRMS assay for the simultaneous determination of eleven (11) candidate cardiovascular drugs.
- To apply the developed microsampling based LC - HRMS assay for multi compound drug determination in volunteer/patient microvolume dried blood samples to indicate adherence to prescription cardiovascular medication. Volunteers who are prescribed one or more of the target cardiovascular drugs will be recruited from De Montfort University staff in Leicester - UK and from cardiovascular disease patients attending a routine clinical follow-up at the Al Sader Teaching Hospital and the Misan Cardiac Centre in Iraq. Dried blood spots and blood microvolume samples from a novel DBS self-collection device will be obtained as finger-prick samples from the recruited volunteers. The collected volunteer samples will be tested using the bioanalytical method developed and validated. For each dried blood sample, the presence of each of the target drug(s) prescribed to the volunteer will be ascertained. The combination of the measured drug concentration, the time at which the last drug(s) dose was taken and average pharmacokinetic data for the target drug(s) would allow medication adherence or nonadherence to be inferred.
- To assess the usability of current microsampling methods for patient self-use. Home sampled or self-sampled DBS approach which provides the patient with the capability to collect their own dried blood samples is the way forward for therapeutic drug monitoring of CVD drugs, since it eliminates the need for phlebotomy appointments or clinic visits which complicates the objective assessment of adherence to medication. Thus, a novel device called volumetric absorptive microsampling (VAMS) developed to overcome the barriers with using the traditional DBS card, for example, for self-sample collection will be used for volunteer sample collection in this research. Hence volunteers will be asked to provide feedback on the ease of use of the VAMS sampler and the DBS card for self-sample collection.

Chapter 2 Drug selection for the medication adherence study

This chapter focuses on the selection of cardiovascular drugs for investigation. The types of cardiovascular drugs commonly prescribed in the UK and the county of Leicestershire are discussed. A compilation of the relevant pharmacokinetic, bioavailability and dosage information for the selected drugs which will influence the detection capabilities required for the investigation is presented. As well as the analytical techniques that have previously been used for the quantification of the cardiovascular drugs in biological matrix such as plasma and serum.

2.1 Introduction

Success in drug discovery research means there are several medicines that can be used to manage cardiovascular disease. Most of these medicines are taken orally as tablets or capsules. Cardiovascular medications are grouped into different therapeutic classes depending on their mode of action in the body. In the UK, several factors are considered by clinicians when selecting a medication for the treatment of a patient's condition. The main ones include safety, efficacy and cost. Prescriptions are therefore normally issued in line with prescribing guidelines provided by the NHS (Collier et al., 2017). The frequency of taking the prescribed medication depends on the patient's condition thus whether acute or chronic. Since cardiovascular disease is a chronic condition, medications must be taking regularly by the patient. Despite the wide variety of cardiovascular medications available for treatment, the goal of proper management of cardiovascular disease with pharmacotherapy is still not achieved in many patients due to nonadherence to treatment (Baroletti and Dell'Orfano, 2010).

The cause of CVD's is multifactorial and involves several risk factors, the most common include: hyperlipidaemia, diabetes and obesity (Gonzalez et al., 2015). The prevalence of any of the risk factors is known to increase with age and lifestyle. The result is an increase in the prevalence of multimorbidity and the total number of medications taken (Stegmann et al., 2010). Hence the need for combination of drugs to properly

manage cardiovascular disease. Cardiovascular disease patients are therefore normally prescribed with cardiovascular medications from different therapeutic classes (complex regimen) (Anderson and Nawarskas, 2001). Combined therapy, ageing and the high rates of multimorbidity leads to polypharmacy prescribing where patients are prescribed with no less than five different medications at the same time (Volpe et al., 2010; Di Bari and Balzi, 2017). Polypharmacy increases the risk of adverse drug effects and drug interactions for patients with cardiovascular disease mainly the elderly (Abolbashari et al., 2017). Drug interactions are further complicated by the regional and pharmacogenetic differences in response to cardiovascular medication (Joseph et al., 2014). All this points to the fact that a one size fits all approach to treatment is not the best. There is therefore the need to look carefully at the levels of cardiovascular drugs in the patient's blood. As demonstrated by (Lawson et al., 2013, Tanna et al., 2015; Bernieh et al., 2017), with the use of DBS based LC-HRMS analyses for the determination of CVD drug. Such data could provide the basis for clinical decision making and help tailor the treatment to each patient.

2.2 Types of cardiovascular drugs prescribed in the UK

There are several classes of cardiovascular drugs prescribed in the UK. The most commonly used are angiotensin converting enzyme (ACE) inhibitors, beta blockers, cardiac glycosides, diuretics, angiotensin 2 receptor antagonists, nitrates, calcium channel blockers, anticoagulants, antiplatelets and statins. Table 2.1 presents examples of the commonly prescribed oral CVD medication in the UK with their respective therapeutic classes.

Table 2.1 Table 2.1 Types of commonly prescribed oral cardiovascular drugs in the UK and their classes (BNF, 2016)

DRUG	CLASS
Digoxin	Cardiac glycosides
Indapamide, chlorothiazide, benzthiazide, metolazone, chlortalidone, clopamide, furosemide, bumetanide, torasemide, butanide spironolactone, eplerenone amiloride, triamterene	Thiazide & related diuretics Loop diuretics Aldosterone antagonist Potassium sparing diuretics
Acebutolol, atenolol, betaxolol, bisoprolol, carteolol, carvedilol, celiprolol, esmolol, labetalol, levobunolol, metoprolol, nadolol, nebivolol, oxprenolol, pindolol, propranolol, sotalol, timolol	Beta blockers
Doxazosin, terazosin, prazosin	Adrenergic neurone blockers
Lisinopril, fosinopril, imidapril, moexipril, ramipril, perindopril, trandolapril, captopril, enalapril, quinapril	ACE Inhibitors
Azilsartan, losartan potassium, candesartan, eprosartan, irbesartan, olmesartan, valsartan, telmisartan	Angiotensin II receptor antagonist
Glyceryl trinitrate, isosobide mononitrate, isosobide dinitrate	Nitrates
Amlodipine, felodipine, nimodipine, lacidipine, nifedipine, isradipine, diltiazem HCL, verapamil,	Calcium channel blockers
Nicorandil	Other anti – angina drugs
Edoxaban, fondaparinux, danaparoid, bivalirudin, argatroban, apixaban, rivaroxaban, dabigatran, Warfarin, acenocoumarol, rivaroxaban,	Anticoagulants
Aspirin, clopidogrel	Antiplatelet drugs
Tranexamic acid	Antifibrinolytic drugs
Atorvastatin, fluvastatin, simvastatin, pravastatin, rosuvastatin, ezetimibe	Statins

2.3 Top 10 cardiovascular drugs prescribed in the UK

One of the objectives of this research as outlined in (section 1.5 of Chapter 1) was to develop a microsampling based bioanalytical assay to identify and quantify selected commonly prescribed cardiovascular medications that a patient may be taking orally. Hence the top most prescribed cardiovascular drugs in the UK were considered.

Cardiovascular drugs for the study were therefore selected based on information acquired on the top ten cardiovascular drugs by the volume of prescriptions issued in

the UK (Table 2.2). As well as data compiled on the commonly prescribed cardiovascular drugs by general practitioners (GPs) and health personnel in the county of Leicestershire (Table 2.3). The idea was to ensure that volunteers undergoing prescribed cardiovascular treatment could be easily recruited within the university community for the planned study.

Table 2.2 Top 10 cardiovascular drugs prescribed in the UK by prescription volume for the year 2013 (Health & Social Care Information Centre, prescription cost analysis 2014)

Number	Cardiovascular drug	Prescription volume (thousands)
1	Simvastatin	39,856.0
2	Aspirin	30,611.0
3	Ramipril	24.939.6
4	Amlodipine	23,074.8
5	Atorvastatin	18,249.9
6	Bendroflumethiazide	16,007.5
7	Bisoprolol	15,957.1
8	Atenolol	10,675.0
9	Lisinopril	10,006.9
10	Losartan	6,940.4

Table 2.3 List of CVD drugs prescribed locally by hospital specialist and GP's in Leicestershire (Leicestershire health community NHS, medicines formulary).

DRUG	CLASS	ORDER OF PREFERENCE
Digoxin	Cardiac glycosides	1st
Indapamide Furosemide Butanide Amiloride	Thiazide & related diuretics Loop diuretics Potassium sparing diuretics	1st 1st 2nd 1st
Atenolol Bisoprolol	Beta blockers	1st 2nd
Doxazosin	Adrenergic neurone blockers	1st
Lisinopril Ramipril	ACE inhibitors	1st 2nd
Losartan potassium Candesartan	Angiotensin II receptor antagonist	1st 2nd
Glyceryl Trinitrate Isosobide Mononitrate	Nitrates	1st 1st
Amlodipine Diltiazem HCL	Calcium channel blockers	1st 1st
Nicorandil	Other anti – angina drugs	1st
Warfarin Rivaroxaban	Anticoagulants	1st 2nd
Aspirin Clopidogrel	Antiplatelet drugs	1st 2nd
Tranexamic Acid	Antifibrinolytic drugs	1st
Simvastatin Atorvastatin	Statins	1st 2nd

The data from the Table 2.2 was compared with Table 2.3 and the following cardiovascular drugs were selected for the study mainly because they are among the most recommended for first line therapy both in the UK and in the county of Leicestershire where the study will be undertaken. They are amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan. The decision was taken to operate the mass spectrometer in the positive ionisation mode, hence from Tables 2.2 and 2.3, aspirin and bendroflumethiazide were not included in the list of selected drugs for investigation because they ionise negatively. Valsartan which does not appear in the list in Tables 2.2 and 2.3 was included for investigation because there was a permanent volunteer

taking valsartan in combination with amlodipine and bisoprolol to provide microvolume blood samples for investigation.

It is envisaged that the drugs belonging to different groups or classes will present a challenge for the development of the dried blood microsampling based LC-HRMS assay. This is because different classes of drugs will have different physicochemical characteristics (molecular weight, logP, logD, pKa, polarity, stability, dissolution, protein binding and thermodynamic properties). For example, atenolol is a beta blocker and lisinopril is an ACE inhibitor but both drugs are hydrophilic, whilst atorvastatin and simvastatin belong to statins and are extremely hydrophobic. Hence the wide difference in solubility could impact on extraction, separation and subsequent quantitation.

2.4 Pharmacokinetic, bioavailability and dose information for the CVD drugs

An objective of this research was to investigate if the presence of the specified target analytes can be quantified in microvolume blood samples from individual volunteers undergoing treatment with oral cardiovascular medication. Therefore, the range in blood drug concentrations from individual volunteers is considered more significant than just an average value for the population because of the regional and pharmacogenetic differences which is known to affect the concentration of drugs in individual patients (Joseph et al., 2014).

For a bioanalytical method to be used to infer adherence to medication, the requirement will be to detect residual levels of drug or its metabolite up to 24 h after the initial dose was taken. From this time point the patient will be taking a repeat dose. In view of this, the minimum likely drug concentration in the dried blood microvolume sample must be taken into consideration. The blood drug levels of the patient will depend on the dosage of medication given, the drug pharmacokinetic (PK) parameters for that patient, bioavailability and the time delay post dosing. No comparison studies have been performed between plasma and DBS concentration levels for any of the selected target drugs and such comparison work could not be

investigated as part of this research. Hence the PK, bioavailability and dosage information derived from plasma sample for the selected target drugs is essential in defining the detection parameters required for this study.

It should however be noted that drug solubility is another important factor that can impact on the desired plasma levels of drug for pharmacological activity. Murtaza (2012) reported that about 40% of oral drugs are not soluble in water and are therefore poorly absorbed from the gastrointestinal tract (GIT) resulting in low bioavailability. Available literature PK data, for the selected target drugs, was gathered to help define the detection parameters required of the analytical instrumentation (Tanna and Lawson, 2014). Table 2.4 shows documented PK information as well as dosage and bioavailability for the eleven target drugs investigated.

To define the calibration ranges for investigation, the lowest possible concentration of each drug in DBS must be taken into consideration. This will be the case for either a drug with a very low bioavailability or a low dosage, rapid elimination (short $t_{1/2}$) with a low C_{max} attained in a short time (t_{max}). In view of this drugs like losartan and simvastatin (Table 2.4) should present the greatest challenge.

Hence Table 2.4 was used as the basis to determine the proposed calibration range for each of the selected drugs considering the C_{max} data reported for the available dosages of each drug, the time it takes to reach the highest concentration (t_{max}) and the duration for the blood drug concentration to reduce by half ($t_{1/2}$). The WHO states that drugs are not effective after about 4.5 half-lives, which is equivalent to a blood drug concentration of <5% of the C_{max} (Moffat et al., 2011). Thus, the calibration ranges for each of the selected drugs were therefore chosen to cover (<2% the C_{max} value of the lowest dose to the respective C_{max} value of the highest dose of that drug (Table 2.4)).

For example, from Table 2.4 the smallest and biggest doses for doxazosin are 1mg and 16mg with a reported C_{max} range of 8 – 14ng/ml and 49 – 84ng/ml respectively. The C_{max} of doxazosin is reached in about 3 hours (t_{max}) and it has a $t_{1/2}$ of about 22 hours. Thus 2% of 8ng/ml which is the low Cmax value of 1mg is 0.16ng/ml. Hence a calibration range of 0.1 – 100ng/ml was chosen to ensure that the target drug could be

adequately quantified in volunteer samples well below and above the therapeutic concentration range of 8 – 84ng/ml for all the available doses of doxazosin. Hence, eliminating the risk of generating false negative results.

Table 2.4 Pharmacokinetic, bioavailability and dosage information for the selected cardiovascular drugs.

Drug	Dose (mg)	Cmax (ng/ml)	T max (h)	T half (h)	Bioavailability (%)	References
Amlodipine	2.5					Abernethy, 2012
	5	5 -7	5 - 8	35 – 50	60 – 80	Bhatt et al., 2007; Meredith, 1992
	10	17.7	6 – 8	40 – 60	60 – 65	Ramani et al., 2009; Lv et al., 2013
	10	11.7 – 14.1	7.5 – 8.3	40 – 60	60 – 80	Nirogi et al., 2006; Qi et al., 2013
						Park et al., 2006,
Atenolol	25					
	50	159 – 377	1.5 – 4	6 – 7, 4 – 11	50	De Abreu et al., 2003; Irshaid et al.,
	100	590 –1370	2 – 4	5 – 8		1996; Najib et al., 2005; Vergin and
						Nitsche, 1989; Wu et al., 2003;
						Lawson et al., 2012
Atorvastatin	10	2.1 - 17.1	0.7 – 3.0	11 – 14	12 – 14	Garcia et al., 2003; Koytchev et al.,
	20	3.2 – 10.6	0.8 – 2.8	7.4 – 20.2		2004; Yacoub et al., 2013;
	40	5.0 – 20.5	0.5 – 3.0	4.2 – 11.4	14	Lennernas, 2003; Ghosh et al. 2011;
	40	27 – 66	2.0 – 3.0	15 - 30	12	Mendoza et al., 2006; Shen et al.,
	80	45.9 – 71.5				2012; Tippabhotla et al., 2013;
						Bellosta et al., 2004
Bisoprolol	1.25					
	2.5					Lancaster and Sorkin 1988; Leopold
	3.75	17.3 – 26.9	1.5–4, 2 – 4	5 – 16, 8 – 14	>90	1986; Bhatt et al., 2007; Leopold et
	5	37–87, 29-43	1.2 – 2.6	7.1 – 9.1	90, 82 - 94	al., 1986; Ding et al., 2007a; Ding et
	10			10 - 11	90	al., 2007b; Liu et al., 2007
Diltiazem	60	21.9 – 86.7	1.8 – 3.86	1.9 – 4.5	40	Hermann et al., 1983
	90	41.7 – 109.3	1.9 – 3.6	3.2 – 4.3	27 - 49	Echizen et al., 1986
	120	98 - 304	1.5 – 4.5	5.1 - 7.9	34 - 54	Boyd et al., 1989
	300	90.3– 113.1	12.1 – 14.2	10.3 – 13.0	50	Quiroga et al., 2001
Doxazosin	1	8 – 14	2 - 3	22	20.5	Kwon et al., 2007; Ma et al., 2007;
	2	13.2 – 23.6	2 – 3	24.7 – 33.7	65	Sripalakit et al., 2005; Sripalakit et
	4	20.9 – 37.7	2.2 – 5.2	10 - 12	65 – 69	al., 2006; Elliot et al., 1987; Chun et
	8	49.2 – 84.4	2.7 – 5.1	14.4 – 26.6	50.3 – 75.3	al., 2006; Chung et al., 1999;
	16					Conway et al., 1993.
Lisinopril	10	20 - 40	6 – 8	12	21 - 29	Gomez et al., 1987; Lancaster &
	20	50 - 88	5.8 – 7.8	12.6	16 - 36	Todd, 1988; Huang et al., 2006;
	80	80 – 140	6 – 9	12	25 - 50	Beermann 1988; Padua et al., 2004;
		52.7 – 121.3				Bendtsen et al 1989; Qin et al. 2007
Losartan	25	43.6 – 125.4	0.5 – 1.1	0.94 – 4.02		Ohtawa et al., 1993,
	50	79 - 433	0.54 – 2.52	1.77 – 2.97	33	Yang et al., 2012; Sica et al 2005
	50	89.1 – 306.1	0.5 – 2.2	0.94 – 2.44	20.3 – 51.3	Ohtawa et al., 1993;
	75	263.6 -783.4	0.54 – 1.88	1.51 – 2.31	25 – 35	Salvadori et al., 2006,
	100	274 – 1036.4	0.4 – 1.0	1.1 – 1.92	33	Ohtawa et al., 1993; Rao et al 2012
Ramipril	1.25					Hosie & Meredith 1991; Kelly &
	2.5	4.7 – 7.7	2 – 4	4 – 6	44 - 66	O'Malley 1990; Meisel et el., 1994;
	5	9.3 – 20.8	0.5 – 1	3 – 7	54 - 65	Van Griensven et al., 1995
	10	11 – 31	1 - 2	4 – 7		
Simvastatin	10	4.9 – 5.86	1.98 – 2.52	1.78 – 3.06	5	Selvan et al., 2009
	10	2.4 – 3.5	1 - 3	2.5 – 6.6		Kosoglou et al., 2002
	20	4.9 - 5.9	2.0 - 2.52	1.8 – 3.06	5	Jang et al., 2010; Patel et al., 2008
	40	5 - 40	1.7 - 3	2.0 – 5.9		Reinoso et al., 2002
	40	1.1 – 4.4	0.6 – 2.9	2.5 – 4.0	<5	Najib et al., 2003
	40	7.4 – 12.2	1.1 – 1.8	3.6 – 6.1	5	Zhi et al.,2003
	40	10 – 34	1.3 – 2.4	2 – 3	5	Bellosta et al., 2004
	40	5 - 38.1	1.2	1.8 – 3.8		Yan et al., 2003
	80	15 – 58.1	2.1 – 2.4	3.5 – 3.6	5	Ahmad et al., 2011
	80	10.34 – 57.4	1.50 - 3	10.8	5	Barrett et al., 2006
Valsartan	20					
	40	1010 - 2270	2 - 4	6 – 9, 6 - 10		Iqbal et al., 2010; Kim et al., 2014;
	80	1200 - 2540	1.5 - 2	5.5 – 8.6	23	Li et al., 2007; Flesch et al., 1997;
	160	1786 – 3460	0.83 – 4.97	3.3 – 5.3	10 - 35	Zakeri-Milani et al., 2010;
	160	1930 - 3940	1.5 – 3.0	4.1 – 6.1		Bindschedler et al., 1997

2.5 Quantification of the selected cardiovascular drugs reported in DBS, plasma and serum

A variety of analytical techniques have been documented for the detection and quantification of the selected cardiovascular drugs in DBS, plasma, serum and urine. These include LC with UV, fluorescence, or MS detection. LC coupled with MS techniques involves LC-MS, LC-MS/MS and LC-HRMS. A comparison of analytical techniques reported in the literature for the determination of the selected cardiovascular drugs showing the calibration ranges investigated and the sample volume used for analysis in DBS, plasma and serum is presented in Table 2.5. Data on published work in urine was not included in Table 2.5, since drug/metabolite levels in urine do not reflect a true measure of the analyte in circulating blood. This is because the urine matrix is linked with the elimination stage of the pharmacokinetic disposition of the target drug in the body under the process of absorption, distribution, metabolism and elimination (ADME).

Despite all the data in Table 2.5, there are only four (4) references reported for the quantification of some of the selected cardiovascular drugs, which include amlodipine, atenolol, bisoprolol, ramipril and simvastatin in dried blood spots (DBS). Thus, affirming the need for the development of a microsampling based LC - HRMS assay for the simultaneous determination of the eleven (11) candidate cardiovascular drugs.

Table 2.5 Analytical techniques documented for the quantification of the selected CVD drugs in DBS, plasma and serum.

Drug	Analytical Method	Calibration Range (ng/ml)	Mobile Phase	Mobile Phase Modifier	Sample Vol (µl)	Matrix	LOQ (ng/ml)	Internal Standard	Ion, Precursor to Product (m/z)	References
Amlodipine	HPLC	10 - 1000	ACN:Acetic acid	0.2% Dihydrogen phosphate	500	Plasma	10	Amytryptyline	None	Alsarra et al., 2009
	LC-MS/MS	0.1 - 50	ACN:Water	0.1% Formic acid	200	Plasma	0.1	Amlodipine d_4	409 - 238	Qi et al., 2013
	LC-MS/MS	0.1 - 10.0	MeOH : (Ammonium formate, 80:20v/v)	0.1% Ammonium formate	1000	Plasma	0.1	Imipramine	409 - 238	Bhatt et al., 2007
	LC-MS/MS	0.2 – 12.8	MeOH: Water 78:22,v/v	0.15% Ammonium acetate	200	Serum	0.2	Omeprazole	409 - 238	Li et al., 2011
	LC-MS/MS	0.2 - 20	Water: MeOH 14:86%, v/v	0.1% Trichloroacetic acid	200	Plasma	0.2	Pravastatin Sodium	408.68 – 238.00	Yacoub et al., 2013
	LC-MS/MS	0.1 - 10	ACN:Water	Ammonium acetate	200	Plasma	0.1	None	409.1 – 237.9	Danafar and Hamidi, 2016
	LC-MS/MS	0.05 - 12	MeOH:NH_4 acetate	Ammonium acetate	500	Plasma	0.05	Gliclazide	409.2 – 238.1	Chan-Mei et al., 2013
	LC-MS/MS	0.2 - 20	MeOH:NH_4 acetate	0.1% Formic acid	1000	Plasma	0.2	Ondansetron	409 - 238	Bathula and Devani, 2011
	LC-MS/MS	0.3 – 20.73	ACN:NH_4 formate	0.2% NH_4 formate	300	Plasma	0.3	Amlodipine d_4	409.2 – 238.1	Jangala et al., 2014
	LC-MS/MS	0.1 – 5.0	ACN:Formic acid	0.1% Formic acid	500	Plasma	0.1	Eplerenone	409.1 – 238.14	Rezk and Badr, 2014
	LC-MS/MS	0.02 - 20	ACN:NH_4 formate	0.1% Formic acid	100	Plasma	0.02	Amlodipine d_4	409.4 – 238.2	Shah et al., 2017
	LC-MS/MS	0.1 – 10.22	Water: MeOH	0.2% Formic acid	200	Plasma	0.1	Fluoxetine HCL	409.1 – 238.1	Shentu et al., 2012
	LC-MS/MS	0.46 - 1000	ACN:Water	0.1% Formic acid	50	Plasma	0.46	Nitrendipine	409.1 – 237.9	Yu et al., 2011
Atenolol	LC-MS	200 - 12000	ACN:Water	0.5% Formic acid	500	Plasma	5	Pantoprazole	267.0	Sridharan et al., 2010
	LC-MS/MS	5.05 - 503	Ammonium acetate: ACN, 15:85 v/v	0.2% Ammonium acetate	250	Plasma	5.10	Carbamazepine	267.3 – 145.1	Kallem et al., 2012
	LC-MS/MS	10-800	MeOH:ACN	0.1% Formic acid	200	Plasma	10	Atenolol- d_7	267.0 – 190.1	Kallem et al., 2013
	LC-MS/MS	1 - 800	MeOH:Water	0.1% Formic acid	500	Plasma	5	Phenazone	267.2 – 190.1	Phyo Lwin et al., 2017
	LC-HRMS	**25 -1500**	**ACN:Water**	**0.1% Formic acid**	**30**	**DBS**	**25**	**Atenolol- d_7**	**267.1903**	**Lawson et al., 2012**
	LC-MS/MS	7.81 - 1000	ACN:Water	0.05% Formic acid	200	Plasma	7.81	Quinoxaline	267.2 – 145.0	De Nicolo et al., 2016

Table 2.5 continued

Drug	Analytical Method	Calibration Range (ng/ml)	Mobile Phase	Mobile Phase Modifier	Sample Vol (µl)	Matrix	LOQ (ng/ml)	Internal Standard	Ion, Precursor to Product (m/z)	References
Atorvastatin	LC-MS/MS	0.035 - 25	ACN: Ammonium acetate, 50:50v/v	0.3% Formic acid	500	Plasma	0.035	Lansoprazole	559.09 - 440.21	Ravi et al., 2012
	LC-MS/MS	0.10 – 30.0	0.1% Formic acid: ACN, 20:80,v/v	0.1% Formic acid	250	Plasma	0.1	Nevirapine	559.2 - 440.2	Ghosh et al., 2011
	LC-MS/MS	1.5 - 150	Water:MeOH, 14:86, v/v	0.2% Trichloroacetic acid	200	Plasma	1.5	Pravastatin Sodium	559.09 – 440.21	Yacoub et al., 2013
	LC-MS/MS	0.2 - 151	MeOH:ACN	0.2% Acetic acid	250	Plasma	0.2	Proguanil	559.2 – 440.0	Gagula et al., 2012
	LC-MS/MS	0.05 - 100	Water:ACN:MeOH, 35:25:40, v/v/v	0.005% Formic acid	200	Plasma	0.05	Atorvastatin-d_5	557.4 -278.1	Partini et al., 2013
	LC-MS/MS	0.1 - 100	ACN : Ammonium acetate, 30:70 v/v	0.1 Ammonium acetate	900	Serum	0.1	Atorvastatin-d_5	559.0 – 440.1	Novakova et al., 2009
Bisoprolol	LC-MS/MS	0.5 -70	ACN:Ammonium formate 5mM, 80:20 v/v	5mM Ammonium formate	500	Plasma	0.5	Metoprolol	326.2 - 116.1	Bhatt et al., 2007
	LC-MS/MS	0.05 - 120	0.1 formic acid: MeOH, 32:68v/v	10mM Ammonium acetate	1000	Plasma	0.05	Metoprolol	326.4	Ding et al., 2007
	LC-MS/MS	0.1-30	1mM Ammonium acetate:MeOH: ACN, 65:17.5:17.5v/v/v	0.1% Formic acid	250	Plasma	0.1	Moxifloxacin	326 - 116	Tutunji et al., 2009
	LC-MS/MS	0.5 -100	MeOH: Ammonium acetate: Formic acid, 70:30:0.1, v/v/v	0.2% Formic acid	100	Plasma	0.5	Diphenhydramine	326 - 116	Liu et al., 2007
	LC-HRMS	1.0 - 500	ACN:Water	0.1% Formic acid	1000	Serum	3.3	Metoprolol D_7	326.2331	Tomkov et al., 2017
	LC-HRMS	**1 -100**	**ACN:Water**	**0.1% Formic acid**	**30**	**DBS**	**25**	**Atenolol- d_7**	**326.2326**	**Lawson et al., 2013**
Diltiazem	HPLC	10 - 200	Ammonium dihydrogen PO_4:ACN, 20:80v/v	0.5mM Triethylalamine	1000	Plasma	2.5	None	N/A	Baviskar et al., 2009
	LC-MS	1 - 225	MeOH:Ammonium acetate	10mM Ammonia	1000	Plasma	1.0	Codeine	415.5	Molden et al., 2003
	LC-MS/MS	0.48-639.9	10mM Ammonium acetate:ACN,25:75,v/v	10mM Ammonium acetate	300	Plasma	0.48	Ziprasidone	415.37 - 177.93	Dasandi et al., 2009
	LC-MS/MS	1 - 200	ACN:Water	0.02% Formic acid	100	Plasma	1.0	Sulfadimethoxine	415.4 – 178.2	Li et al., 2007

Table 2.5 Continued

Drug	Analytical Method	Calibration Range (ng/ml)	Mobile Phase	Mobile Phase Modifier	Sample Vol (µl)	Matrix	LOQ (ng/ml)	Internal Standard	Ion, Precursor to Product (m/z)	References
Doxazosin	LC-MS/MS	0.5 - 100	20mM Ammonium acetate:MeOH:ACN, 55:10:35, v/v/v	20mM Ammonium acetate	250	Plasma	0.1	Terazosin	388	Ma et al., 2006
	LC-MS/MS	0.1 -50	MeoH:Ammonium actetate	0.2% Formic acid	200	Plasma	0.1	Prazosin	452 - 344	Liu et al., 2010
	HILIC-MS/MS	0.2 - 50	ACN:Ammonium formate	0.1M NaOH	100	Plasma	0.2	Cisapiride	452.2 – 344.0	Ji et al., 2008
	LC-MS/MS	0.078-10	ACN:Water	0.05% formic acid	200	Plasma	0.078	Quinoxaline	452.2 - 343.9	De Nicolo et al., 2016
Lisinopril	LC-MS	6 - 150	ACN:MeOH, 72:7:21	Ammonium formate	1000	Serum	6	Hyoscyamine	406.5	Tsakalof et al., 2002
	LC-MS	2.5 - 320	MeOH:ACN, 58:25:17, v/v	0.1% Formic acid	250	Plasma	2.5	Enalaprilat	406	Zhou et al., 2008
	LC-MS/MS	2 - 200	ACN:Water, 60:40,v/v	0.2 % Acetic acid	500	Plasma	2	Enalaprilat	406.3 - 84.3	Padua et al., 2004
	LC-MS/MS	1.29 - 129	ACN:Water, 11:89, v/v	0.1% TFA	250	Plasma	1.29	None	406 - 246, 291, 309	Vlase et al., 2010
	LC-MS/MS	0.5 - 250	ACN	5mM Ammonium formate	100	Plasma	0.5	Lisinopril-d_5	404.3 – 114.1	Shah et al., 2016
	LC-MS/MS	0.78 -100	MeOH:Water, 50:50, v/v	0.1% Formic acid	400	Plasma	0.78	Pseudoephedrine HCL	406.1 – 246.0	Huang et al., 2005
	LC-MS/MS	2 - 200	Ammonium Acetate:MeOH, 70:30v/v	0.2% Acetic acid	500	Plasma	2	Enalaprilat	406 - 246	Qin et al., 2007
	LC-MS/MS	1.03 - 206	MeOH:Water, 55:45,v/v	0.2% Formic acid	100	Plasma	1.03	Enalaprilat	406 -246, 84	Qin et al., 2011
Losartan	LC-MS	1.0 - 1000	ACN:0.2% Formic acid, 68:32, v/v	0.2% Formic acid	200	Plasma	1.0	Butylparaben	422.79	Choi et al., 2008
	LC-MS/MS	4.0 - 800	ACN:0.05% Acetic acid, 70:30,v/v	0.05% Acetic acid	200	Plasma	4	Valsartan	421 - 179	Salvadori et al., 2009
	LC-MS/MS	1 - 1000	Triethylamine:Acetic acid:ACN	0.1% Acetic acid	400	Plasma	1	DuP-167	421.2 – 126.7	Iwasa et al., 1999
	LC-MS/MS	5 - 500	ACN:Water	0.2% Formic acid	50	Plasma	5	Losartan-d3 carboxylic acid	422.9 – 207.1	Jia et al., 2010

Table 2.5 Continued

Drug	Analytical Method	Calibration Range (ng/ml)	Mobile Phase	Mobile Phase Modifier	Sample Vol (µl)	Matrix	LOQ (ng/ml)	Internal Standard	Ion, Precursor to Product (m/z)	References
	LC-MS/MS	2 - 400	ACN:Water	0.1% Formic acid	250	Plasma	2	Irbesartan	420.73 – 126.70	Prasaja et al., 2009
	LC-MS/MS	1 - 500	ACN	0.1% Formic acid	100	Plasma	1	Losartan-d5 carboxylic acid	423 - 207	Polinko et al., 2003
Ramipril	LC-MS/MS	0.1 - 25	ACN:Ammonium acetate 20:80, v/v	5mM Ammonium acetate	500	Plasma	0.1	Trandolaprilat HCTZ	415.43 – 154.97	Gupta et al., 2011
	LC-MS/MS	0.078 - 100	ACN:Water	0.05% formic acid	200	Plasma	0.078	Quinoxaline	417.1 -234.2	De Nicolo et al., 2016
	LC-MS/MS	0.1 - 100	Methanol	0.1% Formic acid	300	Serum	0.1	Enalapril	417.3 – 234.3	Lu et al., 2006
	LC-MS/MS	2 - 170	MeOH:Water	0.1% Formic acid	200	Plasma	2	Carbamazepine	417.2 – 234.1	Patel et al., 2014
	LC-MS/MS	0.25 - 208	Formic acid:ACN, 15:85	0.2% Formic acid	500	Plasma	0.25	Nevirapine	417.2 – 234.3	Pilli et al., 2010
	LC-MS/MS	0.2 - 80	MeOH:Water, 70:30, v/v	5mM Ammonium formate	300	Plasma	0.2	Ramipril-d3	415.3 – 154.1	Tan et al., 2009
	LC-MS/MS	0.107 - 107	Formic acid:ACN, 25:75	0.1% Formic acid	500	Plasma	0.107	Enalapril	417.4 – 234.2	Yuan et al., 2008
	LC-MS/MS	0.5 - 250	ACN:MeOH:Formic acid 4:4:5, v/v/v	0.1% Sodium Phosphate	500	Plasma	0.5	Enalapril	417.2 – 234.1	Zhu el al., 2002
	LC-HRMS	**0.5 - 100**	**ACN: Water**	**0.1% Formic acid**	**30**	**DBS**	**1**	**Atenolol d_7**	**417.2384**	**Lawson et al., 2013**
Simvastatin	HPLC-FD	0.1 - 10	MeOH:Water 80:20, v/v	None	1000	Plasma	0.1	Lovastatin	N/A	Ochiai et al., 1997
	LC-UV	20 - 1000	ACN:Sodium Dihydrogen Phosphate	None	1000	Plasma	20	None	N/A	Carlucci et al., 1992
	LC-MSMS	0.1 - 101	ACN:Ammonium acetate (5mM), 80:20 v/v	5mM Ammonium acetate	500	Plasma	0.1	Nevirapine	419.3 – 285.3	Pilli et al., 2012
	LC-MS	0.1 – 30	ACN:Ammonium acetate(0.5mM)	0.5mM Ammonium acetate	900	Serum	0.05	Simvastatin d_6	419.0 - 199.2	Novakova et al., 2009
	LC-MS/MS	0.2 - 50	ACN:Ammonium formate 80:20,v/v	0.02M Ammonium formate	500	Plasma	0.2	Simvastatin d_6	437.3 – 303.2	Ramani et al., 2009
	LC-MS/MS	0.1 - 20	ACN:0.5% Formic acid, (90:10, v/v)	0.5% Formic acid	200	Plasma	0.1	Propranolol HCL	M+Na+, 441.3 – 325.1	Selvan and Pal., 2009
	LC-MS/MS	0.05 – 20.4	ACN:Water, 85:15, v/v	10mM Ammonium acetate	500	Plasma	0.05	Lovastatin	419.0 - 199.0	Yang et al., 2003

Table 2.5 Continued

Drug	Analytical Method	Calibration Range (ng/ml)	Mobile Phase	Mobile Phase Modifier	Sample Vol (µl)	Matrix	LOQ (ng/ml)	Internal Standard	Ion, Precursor to Product (m/z)	References
Simvastatin	LC-MS/MS	2.5 - 500	ACN:Ammonium acetate	0.5% Acetic acid	475	Plasma	2.5	Lovastatin	419.3 – 199.3	Ahmed et al., 2012
	LC-MS/MS	10 - 10000	ACN: Water, 20:80, v/v	10mM Phosphate	250	Serum	10	Simvastatin d_6	419.2 – 199.1 441.3 – 325.1	Bews et al., 2014
	LC-MS/MS	0.25 - 50	ACN:Water, 75:25, v/v	0.1% Formic acid	200	Plasma	0.25	Lovastatin	M+Na+, 441.3 – 325	Alakhali., 2013
	LC-MS/MS		ACN: 3.0mM formic acid	Sodium acetate	100	Plasma	0.5	Lorvastatin	419 - 285	Jemal et al., 2000
	LC-MS/MS	0.05 - 105.0	ACN:Ammonium acetate	Ammonium acetate	950	Plasma	0.1	Carbamazepine	419.2 – 285.3	Burugula et al., 2013
	LC-MS/MS	0.5 - 500	ACN: Water	Ammonium acetate	250	Plasma	0.5	Simvastatin d3	419.1 – 199.1	Zhang et al., 2004
	LC-MS/MS	0.2 - 100	MeOH:Water	0.1mM Ammonium Trifluoroacetate	500	Plasma	0.1	6-demethyl mevinolin	419.4 – 285.3	Silva et al., 2014
	LC-MS/MS	0.1 - 100	MeOH:Water,	0.1% Formic acid, Ammonium formate	200	Plasma	0.1	Hesperetin	419.23 – 199.13, 285.17, 303.19	Wang et al., 2015
	LC-MS/MS	0.1 -40.0	ACN:5mM Ammonium acetate (82:18, v/v)	0.2% Acetic acid	300	Plasma	0.1	Lorvastatin	419.1 - 199.3	Apostolou et al., 2008
	LC-MS/MS	0.1 - 16.0	ACN:MeOH	Ammonium acetate	1000	Plasma	0.1	Lovastatin	[M + CH3CN + Na]+,481.2	Barrett et al., 2005
	LC-HRMS	**1 - 100**	**ACN: Water**	**0.1% Formic acid**	**30**	**DBS**	**5**	**Atenolol d_7**	**441.2611**	**Lawson et al., 2013**
Valsartan	LC-MS/MS	10,000 - 5,000,000	ACN:Water with 0.01% formic acid	10mM Ammonium formate	500	Plasma	2000	Pravastatin	436.0 - 234.9	Gonzalez et al., 2010
	LC-MS/MS	5.0 - 4000	ACN:MeOH	1% Ammonia	500	Plasma	5	Clonazepam	434.22 – 179.22	Shah et al., 2009
	LC-MS/MS	1.0 - 2000	ACN:Formic acid	0.1% Formic acid	200	Plasma	1	Tamsulosin	434.2 – 179.0	Selvan et al., 2007
	LC-MS/MS	11.7 - 3000	Water: MeOH	None	100	Plasma	11.7	Irbesartan	434.2 – 178.9	Liu et al., 2008
	LC-MS/MS	4.0 - 3600	ACN: Ammonium acetate	0.1% Formic acid	500	Plasma	2	Probenecid	434.2 - 350.2	Li et al., 2007
	LC-MS/MS	2.0 - 2000	MeOH:ACN	0.1% TFA	500	Plasma	5	CGP48791	436.2 – 291.2	Koseki et al., 2007

2.6 Proposed calibration ranges to be investigated for the selected cardiovascular drugs

Except for amlodipine, atenolol, bisoprolol, ramipril and simvastatin (Lawson et al., 2012; Lawson et al., 2013; Tanna et al., 2015), no prior investigations had been carried out on atorvastatin, diltiazem, doxazosin, lisinopril, losartan and valsartan in dried blood matrix. Hence the information from Tables 2.4 and 2.5 for the selected target drugs investigated using plasma and serum was used as the basis to determine the proposed calibration range to be investigated using dried matrix microsampling. The calibration ranges (Table 2.6) were chosen to cover the anticipated levels of each drug in blood from all the recommended prescription doses, given the bioavailability of the drug. For example, bisoprolol has a bioavailability of 90% (Table 2.4) and is giving at a dose of 2.5 – 10mg whilst valsartan has a bioavailability of 23% and is prescribed at a dose of 20 – 160mg, hence the huge difference in calibration ranges of 0.1 – 100ng/ml and 50 – 4000ng/ml for bisoprolol and valsartan respectively. Table 2.6 shows the doses and the proposed calibration ranges selected for the eleven cardiovascular drugs in human whole blood.

Table 2.6 Proposed calibration ranges of the 11 target cardiovascular drugs in human whole blood

Drug	Dose (mg)	Calibration ranges (ng/ml)
Amlodipine	2.5 - 20	0.5 – 100
Atenolol	25 -100	10 – 1500
Atorvastatin	10 - 80	0.5 – 100
Bisoprolol	2.5 - 10	0.1 – 100
Diltiazem	60 - 360	0.5 – 600
Doxazosin	1 - 16	0.1 – 100
Lisinopril	10 - 80	0.1 – 100
Losartan	25 - 100	5 – 1000
Ramipril	1.25 -10	0.1 – 100
Simvastatin	10 - 80	0.1 – 100
Valsartan	20 -160	50 – 4000

2.7 Choice of internal standard

The use of internal standard (IS) in chromatography based bioanalytical assays employing HPLC or LC with (MS, MS/MS and HRMS) has been well accepted and is often considered critical for the performance of the assay in terms of accuracy and precision of analytical results (Piorkowska et a., 2017). The IS will compensate for the associated variability that may occur because of sample preparation, the subsequent extraction of targets from DBS and the possible variability of ionisation.

The application of MS brings along the challenge of matrix effects which may affect ionisation of the analyte at the ion source of the MS leading to variability in analytical results. Hence suitable internal standards may be required that behave similarly to the analyte of interest not only during the extraction and separation but also at the ionisation and detection stages of analysis. The internal standards therefore provide characteristics to compensate for the experimental variability. For the development of DBS based bioanalytical assays, the ideal choice of internal standard is a stable analyte that is readily available and matches the chromatographic properties, recovery and ionisation properties of the target analyte being investigated (Wagner et al., 2014).

On this basis, several types of IS have reportedly been used, examples are close analogue of the target analyte or a stable isotopic label of the analyte. However, for assays developed for clinical applications, the choice of using a close analogue of the target analyte may not be ideal (Srinivas, 2016). This is because, the IS may be a commonly prescribed drug and hence may restrict the wider applicability of the newly developed assay especially in the patient population. It is therefore recommended that a stable isotopic label of the analyte is used when available. The use of stable isotopic label IS efficiently addresses the various issues during extraction, separation and detection allowing for the unambiguous quantification of the analyte of interest. On this basis, atenolol D_7 an isotopically labelled compound was selected as the IS for this study. However, due to the solid nature of DBS samples, it is important to note that unambiguous quantification of the analyte depends on the point of application of the IS. For example, onto the blank sampling paper to dry before blood spotting, in the

extraction solvent, onto the DBS sample prior to punching or spotting separately the blood sample and IS unto the card and punching both into a tube for extraction. Wagner et al (2014) reports that there are arguments on the stage at which the IS should be added in DBS analyses.

Chapter 3 Blood microsampling methods and the various analytical techniques used for the analyses of collected samples

This chapter gives an overview of microsampling methods currently used for blood sample collection. The challenges of using blood microsampling, the process of sample collection and target analyte extraction are discussed. The advantages of using blood microsampling for the assessment of cardiovascular medication adherence and the analytical techniques that are used for analysing dried blood matrix microvolume samples are also presented.

3.1 Introduction

Microsampling involves the collection of small amounts (microlitre – picolitre) volume of samples for analysis of the concentration of an analyte or endogenous substance (Zane and Emmons, 2013). Blood microsampling therefore involves the collection of microvolume blood samples by means of a finger or heel prick. The application of microsampling has evolved innovatively with great emphasis on using the platform to aid clinicians and researchers conduct research in patient populations such as paediatrics, geriatrics and in resource limited areas of the world where traditional liquid blood sampling and storage may be difficult. Microsampling offers enormous advantages in comparison with traditional liquid blood sampling.

3.2 Advantages in using blood microsampling

The benefits of microsampling are numerous and include low blood volume requirement, storage and transportation without special treatment, better analyte stability, enhanced clinical cooperation in clinical trials and reduced unexpected exposure of analysts to biohazard (Zane and Eamons, 2013; Sharma et al., 2014; Nys et al., 2017). These benefits make blood microsampling attractive in areas such as Newborn Screening (NBS), Therapeutic Drug Monitoring (TDM), Pharmacokinetic (PK) and Toxicokinetic (TK) studies, Paediatric studies, Metabolism and pharmaceutical drug

development. This is because of the small volume of blood required for analyses at each sampling time.

For the measurement of cardiovascular drugs from dried blood matrix, the advantages of using microsampling methods are the ease of sampling and minimal invasiveness of the technique (Wilhelm et al., 2014). Microsampling is therefore seen as “patient friendly” due to its high patient acceptability (Zane and Emmons, 2013; Sharma et al., 2014; Verhaeghe et al., 2017). Microsampling overcomes the limitations of traditional blood sampling technique (venepuncture) which is highly invasive and requires relatively large blood volume (1 - 5 ml) at each sampling time to generate sufficient plasma/serum required for analysis (De Nicole et al., 2016). This poses practical challenges in conducting TDM, medication adherence and pharmacokinetic (PK) studies.

For the purposes of drug concentration measurements in TDM and medication adherence, the possibility of self-sampling or “at home” sampling by a patient is unique to microsampling (Spielberg et al., 2000). Samples can be collected at any desired sampling time (Tanna and Lawson, 2014) by the patient and posted by regular mail to the laboratory for analysis (Spooner, 2013). This eliminates the need for appointments, clinic visits and the services of a phlebotomist with huge cost savings (Martial et al., 2016). For example, by means of microsampling, the City Hospital in Birmingham-UK, offers a testing service for Vitamin D for a small fee to clients around the world. A test kit is mailed to the client and samples are taking at home and mailed back to the laboratory for analysis. Results are sent to the client within 5 days by email (City Hospital, Birmingham, 2015). Similar services are provided using DBS microsampling in the USA for the screening of patients at high risk of HIV – AIDS and antiretroviral drug adherence. (Stekler et al., 2017).

Microsampling also allows researchers the possibility to ship dried matrix without the need for dry ice or special equipment at clinical sites compared to liquid blood or plasma (Shah et al., 2013). This presents huge savings in terms of cost of shipment because analytes are more stable in dried matrix compared with liquid matrix

(Waterman et al., 2005; Manicke et al., 2016). For example, for unstable glucuronides, prodrugs and other compounds likely to suffer from interconversion due to enzymatic activity, dried matrix offers a stabilising effect on the compound during sample collection and subsequent shipping to the laboratory for analysis (Sharma et al., 2014). This eliminates the need for adding stabilising agents which may be required for liquid samples such as plasma, serum or whole blood.

Microsampling also enables TDM and medication adherence studies to be performed in resource limited areas of the world where special storage and transportation of blood and frozen plasma samples may not be readily available. For example, in Africa DBS microsampling has enabled the collection of blood samples in remote villages with no access to clinical facilities. Samples are then sent to hospital laboratories in the city for the screening of Human Immunodeficiency Virus (HIV) and assessment of adherence to antiretroviral medication (Boillot et al., 2016).

The ease of usage of microsampling coupled with the capability of modern analytical instruments to quantify ultra-low levels of drugs and/or metabolites has increased its adoption for TDM and for medication adherence studies. This provides a user-friendly means of monitoring medication levels in the body which would otherwise be difficult to do using venepuncture. However, for TDM the gold standard matrix is either whole blood or plasma sample. The reason is to ensure that the sample accurately represents the analyte in circulating blood (Verhaeghe et al., 2017).

3.3 Current microsampling techniques

Even though DBS card microsampling has not been able to meet all primary expectations such as patient self-sampling, collection of fixed volume blood samples or adoption in regulated bioanalysis, it has prompted research into alternative microsampling platforms (Verhaeghe et al., 2017). This has led to the development of other techniques, for example capillary microsampling (CM) (Jonsson et al., 2012), and volumetric absorptive microsampling (VAMs) (Deniff and Spooner, 2014). Each of these developments have been introduced to overcome the limitations observed with traditional DBS card microsampling such as the volumetric hematocrit effect. Though

significant progress has been made in this area, research is still ongoing to develop user friendly self-use devices for both clinical and non-clinical applications. The reason is that microsamplers provide a cheap and easy alternative to collect and store biological specimens from humans (infants, children and adults). Currently, microsampling techniques used include card or paper microsampling (DBS), volumetric absorptive microsampling (VAMs), and capillary microsampling (Stove, 2015).

3.3.1 Card microsampling

Card microsampling refers to the collection of drops of blood on filter paper, popularly known as DBS. Dried blood spot dates back to the 1960's when Dr. Robert Guthrie used the technique to measure phenylalanine in newborns for the detection of phenylketonuria (Shah et al., 2013). Dried blood spot became popular and was utilised mainly in NBS because of the small sample volume. In addition, the filter paper was cheap, readily manufactured, easily printed and have good adsorption properties (Pelton, 2009). Since then cards used for blood sample collection have been commercially available and can be grouped into two types (untreated and chemically treated cards) (Wagner et al., 2014). The card material may either be made of cellulose (Whatman 903, Ahlstrom 226) or non-cellulose based material (Agilent bond elut dried matrix card, (Tomtec PDMS 7 polyester cards, refer to Figure 7.3b)).

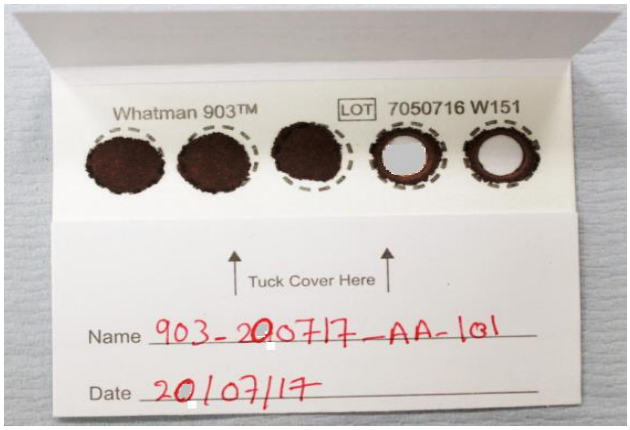


Figure 3.1 A Whatman 903 card containing three spots of dried blood and two punches in the marked sections.

The card thickness, pore size and particle retention power are the main factors that determine the loading capacity and spreadability of blood on the filter paper (Quraishi

et al., 2013). The Clinical and Laboratory Standards Institute (CLSI) recommend the use of three specific sampling cards (Hannon et al., 2007; Wolff, 2017). These are the Perkin-Elmer 226, Ahlstrom 226 and Whatman 903 sampling cards, which serve as the conventional devices for blood sample collection. These types of cards have been used since the inception of the DBS platform in the 1960's. These cards consist of untreated DBS paper mainly cellulose and made from pure cotton linters. They are registered by the U.S. Food and Drug Administration (FDA) as invitro Class II medical devices and approved by the Newborn screening Quality Assurance Program (NSQAP) and the Centres for Disease Control and Prevention (CDC) (Wayne, 2013; Wolff, 2017).

The choice of sampling card is important to attain good quality data, because of the small sample volumes. Conventionally DBS samples collected on a card are analysed by punching a fixed size disk from the spot and extracting with solvent. Hence the size of the disk punched will be directly proportional to the volumetric measure obtained from quantitative analysis (Lawson et al., 2013). The exact blood volume on the punched disk will be a function of thickness of the spot and the area of the disk. It is reported that due to the difference in hematocrit range for individuals, blood from different people will have different viscosities which will affect the spread on cellulose based paper leading to spots of different sizes (Fan and Lee, 2012). This could present variability in analyte quantification due to possible blood spot inhomogeneity, variability in blood spot volume and hematocrit values. These challenges have been overcome with the development of novel devices like VAMS which utilise precise volume sampling or whole spot sampling from capillary collection. Other developments include the HemaSpot – HF blood collection device, Noviplex cards, Hemaxis – DB blood collection device, Ahlstrom 167L cards and Tomtec dry media spot slides which are discussed in Chapter 7.

3.3.2 Volumetric absorptive microsampling (VAMS)

Volumetric absorptive microsampling, an example of which is known as Mitra^{TM} is a microsampling device made of a polymeric tip attached to a moulded plastic handle. The process of sample collection involves the absorption of a liquid sample onto the

porous substrate by wicking. VAMS is a novel development for acquiring a dried blood sample for quantitative analysis. It enables accurate and precise collection of a 10µl or 20µl fixed volume of blood directly from the finger eliminating the volumetric hematocrit effect associated with the conventional DBS sampling when a punched disk is used. Since a precise volume is taken, the whole sample is used for extraction. VAMS come in a clamshell containing two or four sampling devices, which can be closed to securely protect the sample after collection and allowed to dry. There is a label on the clamshell for collecting sample information. Figure 3.2 shows an unused (white tip) and used (red tip) novel VAMS device.

Figure 3.2 VAMS consisting of a hydrophilic polymeric tip attached to a moulded plastic handle. The white-tipped sampler is unused while the red-tipped sampler contains a sample of dried blood (Denniff and Sponner, 2014).

3.3.3 Capillary microsampling

Capillary microsampling (CMS) provides an alternative to collecting a predefined low volume of blood in commercially available glass capillaries (Coleman et al., 2017). These capillaries come in different sizes with volumes in the range of 1–75µl (Nilsson et al., 2013). Example, the Aqua-Cap sample collection tube (Drummond Scientific, USA) designed to be a single-use disposable glass EDTA-coated capillary, which collects precise 75µl of blood (Bowen et al., 2013). Capillary microsampling offers handling of microvolume blood samples in the liquid state from which plasma can be generated through centrifugation of the capillary. This makes CMS compatible with existing work flows for sample handling and clean-up prior to instrumental analysis. Alternatively,

the blood in the capillary can be washed out generating a diluted sample that is then subjected to extraction and analysis or frozen as liquid, or can be applied to absorbent paper to prepare dried blood spots depending on the application required (Parker et al., 2016). Verougstraete et al (2016) reported the use of capillary microsampling as an alternative to traditional venous sampling for the assessment of HbA1c in diabetic patients. It is progressively replacing traditional, larger volume sampling in the assessment of HbA1c in diabetic patients.

It has been demonstrated that, fixed volume blood sample collection using a capillary onto a DBS card eliminates the Hct effect. (Youhnovski et al., 2011; Li et al., 2011; Meesters et al., 2012; Rincon and Meesters, 2014). However, the main benefit of a procedure for a punch DBS analysis is the ease of self-sampling by the patient, which becomes complex and prone to sampling errors when precise blood volumes must be collected by the patient and applied on the DBS card by means of a capillary. This problem has been addressed by the introduction of a novel microsampling device like the Hemapen (Trajan Scientific and Medical, 2015). Designed in the form of a pen to sample precise volumes of blood with ease using capillary collection onto a precut DBS card housed in a secured cartridge within the device. Thus, eliminating the Hct effects and more importantly offering the possibility of self-sampling by the patient. A description of the Hemapen is shown in Chapter 7, Section 7.1.6.

3.3.4 Blood sample collection, spotting and storage on DBS and VAMS

The popularity of microsampling stems from the fact that, collection of sample is taken from a finger (adults) or heel prick (children) rather than a venepuncture. This makes it minimally stressful for the patient. Microsample blood collection involves a sampling kit, containing a sampling device (DBS card, VAMS, small vacutainers), a disposable sterile lancet, gauze, capillary tubes or pipetting device, envelopes, desiccant, and zipper storage bags for shipping.

For clinical applications, capillary blood collection is the conventional approach to microsampling. Microsamples are obtained from each volunteer by gently massaging the fingertip to encourage blood flow. The finger is pricked with a retractable lancet

and the first drop of blood wiped with a sterile gauze. Subsequent drops are either deposited onto marked sections on a sampling card or wicked onto the tip of a VAMS device and labelled appropriately. In the case of DBS, spot sizes should be sufficient to allow the use of a punch device without compromising the DBS sample. Samples of smaller spot sizes should be rejected. The collected samples are air dried at room temperature for about 3 hours and placed in zipper bags for storage.

The United Nations International Children's Emergency Fund (UNICEF) and the World Health Organization (WHO) certify the quality of card microvolume samples through the provision of standard operating procedures (SOP) and guidance manuals for sample collection (WHO, 2005; UNICEF, 2009). Several sampling protocols have also been published by Public Health Authorities like the NHS in the UK, and the Centre for Disease control and Prevention in the US which describes the process of bleeding, spotting, drying and packaging of samples in newborn screening programmes (CDC, 1993; NHS, 2016).

3.4 Analytical challenges in using dried matrix microsampling

Microsampling since its inception was initially used for qualitative analysis, for example in newborn screening (NBS) programmes for inborn errors of metabolism. Its application outside NBS was limited due to the small sample size, matrix complexity and the lack of sensitive and selective detection techniques. This presented analytical challenges, which hampered the attractiveness of the technique. However, in recent years microsampling has become popular due to the advancement in analytical instrumentation such as LC and MS (Wilhelm et al., 2014). This has allowed the quantification of analytes in biological specimen such as blood collected as microvolume samples.

Irrespective of the numerous advantages, collection of microvolumes of blood on DBS card present challenges for implementation in everyday use. This is to do with the ability to recover the analyte from microsamples. Interferants from the card material and blood matrix, sometimes interfere with MS analysis. Studies have also shown that paper/sample combination may also age and prevent the recovery of analyte with

long-term storage (Wagner et al., 2014). Hence, the gain in analyte stability for some target compounds are sometimes offset by the difficulty encountered in recovering other compounds from the card (Linder et al., 2016).

The volumetric hematocrit effect has also been shown to be a significant parameter that impacts quantitative analyses with card microsampling method (Deniff and Spooner, 2010; De Vries et al, 2013). It has been shown to affect DBS drying time, homogeneity, diffusion and assay reproducibility (Timmerman et al., 2011). The hematocrit (Hct) level represents the relative volume of red blood cells (RBC) in blood. It affects the blood viscosity and that in turn influences the spread of a blood spot (Koster et al., 2015). Blood with high Hct (due to the high cellular composition) is more viscous and leads to the formation of small spots on DBS cards. Hence, the Hct effect is profound when a punch is used (0'Mara et al., 2011). The Hct range varies according to age for healthy adult males and females over the age of 18. It is 40 – 54% and 36 – 48% respectively (Walker et al., 1990). Hct values may however, deviate from these ranges in certain disease states where the relative volume of RBC in blood is affected e.g. anaemia and polycythaemia, which is a rare condition in which the bone marrow cells produce too many red blood cells.

It has been demonstrated that, the approach of whole spot analysis or pre-cut disks eliminates the Hct effect. (Youhnovski et al., 2011; Li et al., 2011; Meesters et al., 2012; Rincon and Meesters, 2014). The problems about the Hct effect have been addressed by the development of novel microsampling devices like VAMs, the Hemapen device and the Tomtec dry media spot slides which have been designed to sample precise volumes enabling the use of whole sample or pre-cut disks for analysis hence eliminating the Hct effects (Deniff and Spooner, 2014; Trajan Scientific and Medical, 2015; Tomtec Limited, 2015). A discussion of the innovations in microsampling methods is presented in Chapter 7. Unfortunately, official guidelines for validation of assays developed using microsampling have not yet been provided by regulatory authorities. Hence for microsampling to be adopted in a regulated environment, it is imperative that these factors are investigated to provide assurance that the analytical results are acceptable (Fan and Lee, 2012).

Influence of the matrix on DBS analyte recovery is important. Some analytes may be difficult to extract from the card matrix due to the formation of complexes with endogenous compounds present in the DBS matrix (Koster et al., 2009; Koster et al., 2013; Vu et al., 2014). Hence the extraction efficiency may be influenced by factors like nature of the DBS material, analyte stability, extraction conditions, analyte solubility, Hct and analyte concentration. The bioanalyst should be aware of these possible interactions to acknowledge them in an early stage of the assay development. For example, in the determination of clarithromycin and rifampicin from DBS, iron from the blood was found to interact with rifampicin to form a complex (Vu et al., 2014). Deferoxamine and ethylenediaminetetraacetic acid (EDTA) were added to the extraction solution to form a complex with the iron and recovery of rifampicin in the DBS extraction increased to approximately 100%. Lawson et al (2012) also demonstrated that in the extraction of captopril from calibration and volunteer DBS samples, captopril was found to undergo rapid oxidation to inactive disulphide dimers. This conversion is known to occur “in vivo” and was found to occur “in vitro” as well during sample preparation and analysis. Hence 1,4-dithiothreitol was used both to pre-treat the DBS cards and as part of the extraction process to ensure stability of the captopril extract from DBS.

3.4.1 Extraction of target analytes from dried blood matrix

The process of DBS extraction can be performed on-line by direct extraction or off-line by punching DBS spots into tubes or well plates before extraction with solvents (Heinig et al., 2011; Oliveira et al., 2014a). On-line approaches involve automated handling of dried matrix samples without any treatment or manual disk punching (Manicke et al., 2016; Damon et al., 2016). Examples of on-line extraction includes flow-through elution of DBS and direct extraction from the surface of the card (Fingerhut et al., 2014; Oliveira et al., 2014b; Griffiths et al., 2015; Verplaetse and Henion, 2016).

For off-line extraction, punching can be done manually or by automation which is now integrated into current workflows (Wong et al., 2010). Biosamples are complex mixture of constituents, with the analyte of interest normally at a lower concentration

compared to other constituents (Gjelstad and Pedersen-Bjergaard, 2014). Hence a clean-up step to remove unwanted material from the sample or a process of selective extraction of the analyte(s) from the blood and/or card matrix prior to concentration and analyses is necessary (Liu et al., 2014; Bylda et al., 2014; Tanna and Lawson, 2016). In addition, the sample volume required is very small, hence the extraction protocol should be robust to guarantee analyte detection and increase recovery. Dried blood spots are solid samples hence they must be extracted with a suitable solvent to enable analysis with commonly used analytical techniques such as LC-MS. The dominant extraction technique for the extraction of analytes from dried blood matrix (DBS and VAM's) is solid liquid extraction (SLE) (Cape et al., 2017).

In solid-liquid extraction, there is a solid phase (the sample) and a liquid phase (the extraction solvent). Three factors; solubility of the target analyte, matrix effects and mass transfer (Alkhateeb, 2015) affect the efficiency of SLE. Solubility of the analyte of interest depends on the type of extraction solvent, which is in turn affected by temperature and pressure. Mass transfer deals with the extraction of the analyte from the core of the card matrix into the solvent. Mass transfer is difficult in SLE because it requires solvent penetration of the solid matrix followed by displacement of the analyte from the adsorbed sites. It is dependent on the particle size, matrix structure and the diffusion of the analyte of interest into the extraction solvent (Singleton, 2012). Hence smaller particle size, high diffusion and solvents with low viscosity facilitate mass transfer. Matrix effects involve the influence of the sample matrix on the recovery of the analyte of interest. For dried matrix samples, components of the blood and the card material may impact negatively on the extraction efficiency of the target analyte (Takyi-Williams et al., 2015).

Some target analytes are also highly bound to proteins which are normally retained on the card, hence co-precipitation can arise during extraction from dried matrix. In such situations, a volatile acid or base such as formic acid (FA) or ammonium hydroxide (NH_4OH) may be used to hydrolyse the drug-protein bond, to increase the extraction and recovery of the target analyte (Cape et al., 2017). Following extraction, the resulting supernatant or extract is subjected to analysis.

3.5 Analytical techniques used in conjunction with dried matrix microsampling

Since the introduction of microsampling various analytical techniques have been used for the identification and quantification of the target analyte. These include immunoassay methods, polymerase chain reaction (PCR) amplified DNA analyses, high performance liquid chromatography (HPLC), and hyphenated techniques such as gas chromatography mass spectrometry (GC-MS), liquid chromatography coupled with mass spectrometry methods (LC-MS, LC-MS/MS, LC-HRMS) (Tanna and Lawson, 2011). However, some of these methods suffer in terms of sensitivity and selectivity. For example, a major drawback for immunoassays is the lack of selectivity due to cross-reactivity of interferents (Tanna and Lawson, 2016; Cape et al., 2017).

In recent years, improvements in bioanalytical detection have allowed for measurement of analytes from blood samples collected using microsampling methods. Such advancement in instrumentation has provided broader options for analysis and improved throughput. This has enabled the extension of microsampling to non-newborn screening applications such as TDM (Kneepkens et al., 2017; Weber et al., 2017), PK and TK studies (Kole et al., 2017), forensic applications (Chepyala et al., 2017; Simoes et al., 2017), sports doping screening (Verplaetse and Henion, 2016; Tretzel et al., 2016), environmental analysis (Provatas et al, 2017), food safety (Xue et al., 2016), endocrinology and metabolism (Heussner et al., 2017).

In a review by Zakaria et al (2016), considering the advantages and challenges of DBS microsampling analysis by MS, they identified 121 biological markers that have been screened using DBS microsampling platform beyond its application in NBS. Out of the 121 biomarkers, 77 were pharmaceutical agents, 6 on illicit drugs, 4 on banned substances in sports and 2 were on chemical exposure agents. This highlights the wide applicability of the dried matrix microsampling platform which has been made possible with advancement in detection technologies notably MS. Many instrumental and methodological solutions specifically dedicated to dried blood microsampling, have now been developed with their common denominator being the use of MS (Wagner et

al., 2014). Making MS the key technique reported in the literature for analysis using microsampling (Zakaria et al., 2016). The reason is because MS provides data which is characteristic of the analyte. The first application of MS to DBS analysis was reported in 1976 for fatty acid determination using direct chemical ionisation (Wagner et al., 2014). Since 1976, MS based bioanalytical assays using microsampling platforms have evolved. Sample collection, preparation, chromatography methods, ionisation sources and mass analysers continue to advance to overcome the challenges associated with using microsampling, as well as meet the needs of the bioanalytical industry (Rago and Negahban, 2016).

3.5.1 Immunoassay

Immunoassays can quantify a wide group of compounds from small molecule therapeutic drugs to biological markers of diseases (Flores et al., 2017). Immunoassay methods have been widely used for the quantification of therapeutic drugs since the 80's, developed in parallel to chromatography methods. Before the adoption of MS detectors in bioanalytical work flows, immunoassay-based methods occupied, the sensitivity gap seen with chromatographic assays (Cape et al., 2017). Hence many new drug applications (NDAs) at the time used bioanalytical methods that were immunoassay based. For example, NDA for lisinopril, a commonly prescribed antihypertensive drug was supported with immunoassays (Breidinger and Woolf, 2017). DBS based immunoassay methods were reportedly used to quantify concentrations of therapeutic drugs irrespective of the small sample volumes (Li et al., 1986). They are easy to perform, require minimal sample preparation with fast analysis time. However, the assay protocol may demand handling radioactive materials, the need for prolonged incubation, selection of the correct antibody, addition of precise volumes of liquids followed by washing (Shipkova et al., 2017). Specificity is based upon the interaction between the analyte and an antibody to the analyte. The disadvantage of such methods is the susceptibility to cross-reactivity by endogenous components and nonspecific binding which leads to a lack of specificity and inability to simultaneously measure different analytes. Hence an immunoassay requires appropriate reagent; that is, a specific antibody for the analyte of interest. Otherwise

there will be inconsistencies between patient results as well as reference ranges caused by susceptibility of the antibodies to interferences or cross-reactions by the structurally related compounds (Leung and Fong, 2014). Currently, LC-MS/MS methods have largely eradicated the need to develop immunoassays to support small molecule bioanalysis using dried matrix microsampling. In contrast, immunoassay methods are still widely used for the measurement of proteins and peptides in biological fluids.

3.5.2 High performance liquid chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is a form of column chromatography that pumps a sample mixture in a solvent (mobile phase) at high pressure through a column with packing material (stationary phase). As the mobile phase passes through the column, the analytes interact with the two phases at different rate due to their polarities (Snyder et al., 2012). Analytes that have less interaction with the stationary phase or the most interaction with the mobile phase exit the column quicker. The components of a typical HPLC system is shown in Figure 3.3.

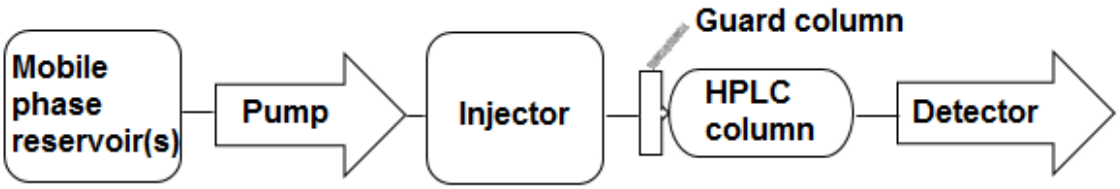


Figure 3.3 A schematic representation of an HPLC system

Chromatographic assays improved specificity by means of separation before detection. Early chromatography based assays used gas chromatography (GC) for separation, but GC was limited because of the requirement for the analyte to be volatile (Stevenson, 2011). This limitation triggered the adoption of HPLC for small molecule bioanalysis, since it eliminated the need for the analyte to be volatile. However, early HPLC assays had a huge limitation in terms of sensitivity. Commonly used HPLC detection method was ultraviolet (UV) spectrometry, which limited sensitivity to the extinction coefficient of the analyte based on the detection wavelength (Suyagh et al., 2010; Stevenson, 2011). Sensitivity problems were partly addressed by the development of

derivatisation procedures to attach chromophores to the analyte which could then be detected by fluorescence (Synder, 2012). DBS based HPLC-UV and fluorescence methods have been used to quantify therapeutic drugs, however these assays suffered in terms of sensitivity. Rao et al (2011) reported DBS based HPLC fluorescence detection for the determination of antihypertensive drugs valsartan and losartan with LOQ's of 120ng/ml and 60ng/ml respectively. AbuRuz et al (2006) reported a DBS based HPLC-UV assay for the TDM of metformin with an LOQ of 150ng/ml. Green et al (2002) also reported a DBS based HPLC assay for the determination of sulfadoxine and pyrimethamine with sensitivities at 10,000ngml and 1,000ng/ml respectively. Allanson et al (2007) also reported a DBS based HPLC-UV method for the quantification of rifampicin with an LOQ of 1,500ng/ml. HPLC sensitivity issues were overcome by the introduction of LC coupled with MS detectors (LC-MS). The MS detector provides the most definitive identification of all HPLC detectors since it provides data characteristic of the analyte (Breidinger and Woolf, 2017).

3.5.3 Liquid chromatography – mass spectrometry (LC-MS)

Over the last two decades, LC-MS has been the main tool for quantitative and qualitative bioanalysis (Xie et al., 2012). LC-MS central position in bioanalysis stems from the fact that there is the need for rapid, effective and sensitive qualitative and quantitative determination of biomarkers to aid drug discovery, TDM, PK and TK studies, neonatal screening and metabolism. LC-MS operating in the single ion monitoring (SIM) mode, assay specificity is partly achieved by chromatographic methods through the separation of analytes prior to detection (Figure 3.2).

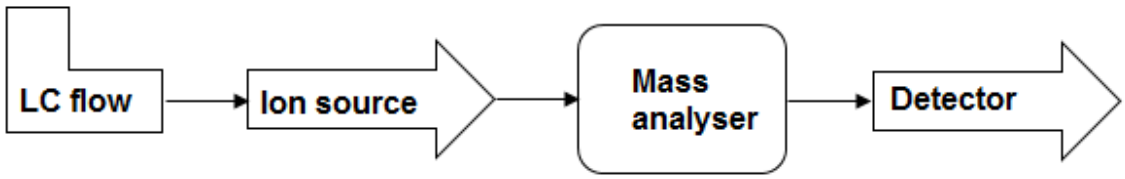


Figure 3.4 A schematic representation of a typical LC-MS system

LC-MS utilises a single mass analyser (the quadrupole mass filter) which has a mass range of around 3,000 with a resolution up to 3000. The quadrupole consists of four

rods with the opposite pairs connected electrically. When a voltage is applied, ions of a particular mass to charge (m/z) follow a stable trajectory through the rods and reach the detector. The quadrupole mass filter is a low resolution device, because it is only capable of measuring the m/z ratio of an ion to the nearest integer value and therefore unable to provide the elemental composition of an ion (Breidinger and Woolf, 2017). LC-MS also utilises soft ionisation which primarily produces molecular species with no fragmentation of the molecule. It is therefore unlikely that the molecular weight alone will make structural assignment possible. Hence the wealth of data resulting from fragmentation pattern from which the molecule can be independently identified is not available. This presents a challenge since there may be several compounds with the same mass to charge ratio (m/z) as the analyte of interest (Lawson et al., 2012). Therefore, in the absence of any data on fragmentation pattern, dependence on retention time will not be enough to provide the needed selectivity. The problem has been overcome with the introduction of tandem mass spectrometry (MS/MS) instruments (Tanna and Lawson, 2016).

3.5.4 Liquid chromatography – tandem mass spectrometry (LC-MS/MS)

Challenges in performing analysis with microvolume dried blood samples forces the bioanalytical scientist to look for better technology that offers advantages in selectivity, sensitivity and robustness. One such technique is LC coupled with MS/MS. The benefits of this technique which include ease of use, lower limits of detection and high throughput are well documented (Li et al., 2014). As a result, LC-MS/MS has rapidly moved from research sites to routine laboratories, gaining reputation in laboratory medicine due to its application in many domains like therapeutic drug monitoring (TDM), newborn screening (NBS), toxicology and drug discovery. The expanding role of LC-MS/MS in DBS analysis is based on improvements in analytical instrumentation which offers unique specificity, faster method development, simultaneous analysis of multiple drugs and their metabolites in microsamples and within a short period of time (Zakaria et al., 2016).

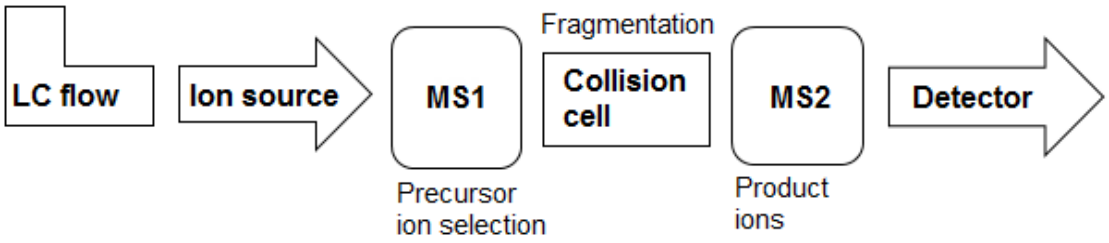


Figure 3.5 A schematic representation of an MS/MS architecture

Tandem MS architecture simply comprises two mass analysers (MS1) and (MS2) with a collision cell in between (Figure 3.5). It initiates the process of fragmentation by allowing only the ions pre-selected by (MS1) into a collision cell, where collision induced dissociation occurs, and the product ions are monitored by (MS2). Tandem MS can be performed by four different configurations of a mass spectrometer (MS). These are triple quadrupole (QqQ), Quadrupole – Time of Flight (Q-ToF), Ion Trap - Time of Flight (IT-ToF) and the Ion Trap. The QqQ, Q-ToF and IT-ToF are examples of tandem in space instruments whilst the ion trap is a tandem in time system (Tanna and Lawson, 2011). The Q-ToF instrument utilises a linear quadruple as MS1 and a Time of Flight (ToF) mass analyser as MS2. The third variant of the linear systems (IT-ToF) has an Ion Trap as MS1 and utilises a ToF as MS2. In an alternative mode of operation an ion trap can reproduce similar fragmentation processes by first isolating ions of a single mass, then relaxing the storage conditions to allow fragmentation to occur and finally analysing the masses of the resultant ions (Johnson et al., 1990). The most frequently cited MS platform in bioanalytical applications is the triple quadrupole (QqQ) mass analyser. This establishes, LC coupled with a QqQ-MS as the gold standard in regulated quantitative bioanalysis (Wagner et al., 2014; Schultz and Henion, 2017).

For DBS analyses, the review by Zakaria et al (2016), shows that out of the 87 drugs analysed by LC-MS methods from the literature, 67 were performed by LC-MS/MS, with drug monitoring for both therapeutic and toxicological studies being the most extensively reported. However, it should be noted that, this does not guarantee LC-MS/MS provides superior advantage and reliable results over other MS techniques (Shipkova and Svinarov, 2016). In contrast with other techniques such as LC-HRMS, LC-MS/MS requires that optimised MS/MS parameters such as (selection of the

appropriate precursor – product ions, declustering potential (DP) and optimised collision energy (CE) needed for collision induced dissociation (CID)) are determined for each compound of interest to be analysed (Zhang et al., 2009; Li et al., 2011). In addition, since only pre-selected ions from a sample by (MS1) enter the collision cell, data on other ions in the sample will be lost hence there is no possibility to revisit the collected data to mine for information on other ions when it becomes relevant. Current LC-HRMS instruments (Bowen et al., 2016) resolve most of these challenges.

3.5.5 Liquid chromatography – high resolution mass spectrometry (LC-HRMS)

Though LC-MS/MS has been established as the standard technique for the development of bioanalytical assays, there has been huge interest within the bioanalytical community in other MS approaches to handle bioanalytical challenges over the past few years (Zhang et al., 2009; Kaufmann et al., 2011; Korfmacher, 2011; Tanna and Lawson, 2011). One such alternative to the traditional LC-MS/MS has been the use of LC coupled with high resolution MS (LC-HRMS). HRMS simply refers to a mass analyser with a resolving power (R) > 10,000 (Xian et al., 2012). For decades, HRMS was mainly used qualitatively in drug metabolism and metabolite identification studies (Ramanathan and Korfmacher, 2012), the reason was due to the poor sensitivity on older variants of HRMS instruments. However, HRMS capabilities for quantitative analysis has recently been established through advancement in instrumentation. Advantages such as accurate mass determination (superior mass accuracy) where the mass of the molecular ion can be measured to better than 1.0 ppm of the relative molecular mass (RMM). Hence, this precise value can be used to determine the atomic composition based on for example, C = 12.0000, H = 1.0078, N = 14.0031, O = 15.9949 and therefore the likely molecular structure (Tanna and Lawson, 2011). For example, considering three compounds of mass 266.3 where atenolol a beta blocker ($C_{14}H_{22}N_2O_3$) = 266.3361, dienestrol ($C_{18}H_{18}O_2$) = 266.3340 and leptospermone ($C_{15}H_{22}O_4$) = 266.3330, nominal mass measurements cannot differentiate these but measurement to the 4th decimal place will distinguish between these compounds. Enhanced compound selectivity (an example of which is demonstrated with atenolol in

Chapter 5, section 5.2.1), full scan acquisition that allows quantification, profiling and data mining post acquisition has given HRMS the competitive edge (An example of which is shown in Chapter 4, Figure 4.1 and 4.2). Rochat et al (2012) argues that a paradigm shift was taking place and HRMS will soon be the first choice in MS detectors.

There are several HRMS platforms available to bioanalysts, these are (Q-TOF (Figure 3.6), TOF, Fourier transform ion cyclotron resonance and orbitrap-based mass analyzers), (Sturm et al., 2016). HRMS has been investigated at research laboratories for the data rich information it can provide in a single run and is now utilised to resolve most of the challenging bioanalytical demands in various fields (Zhang et al., 2009). For example, its ability to conduct multiple drug and metabolite profiling (Ma et al., 2013; Ramakrishnan et al., 2016) by rapid and simultaneous measurements from a single run. This significantly augments the value of data acquired leading to patient care improvement. Simultaneous analyses of multi compounds reduces the sample volume required in comparison to other techniques (Shipkova and Svinarov, 2016). Its capability to reduce method development time with a single MS scan that offers both qualitative and quantitative data is of great advantage (Xie et al., 2012). This has been demonstrated with the analysis of biofluid collected as DBS samples in early drug discovery (Korfmacher, 2011; Ramanathan et al., 2011; King et al., 2014; Korfmacher and Ramanathan, 2016), therapeutic drug monitoring (Li et al., 2011; Oliveira et al., 2014) and medication adherence studies (Lawson et al., 2012a; Lawson et al., 2012b; Lawson et al., 2013; Tanna et al., 2015; Bernieh et al., 2017), environmental science (Krauss et al., 2010). Figure 3.6 shows a schematic of the LC-QTOF-HRMS used for analyses in this study.

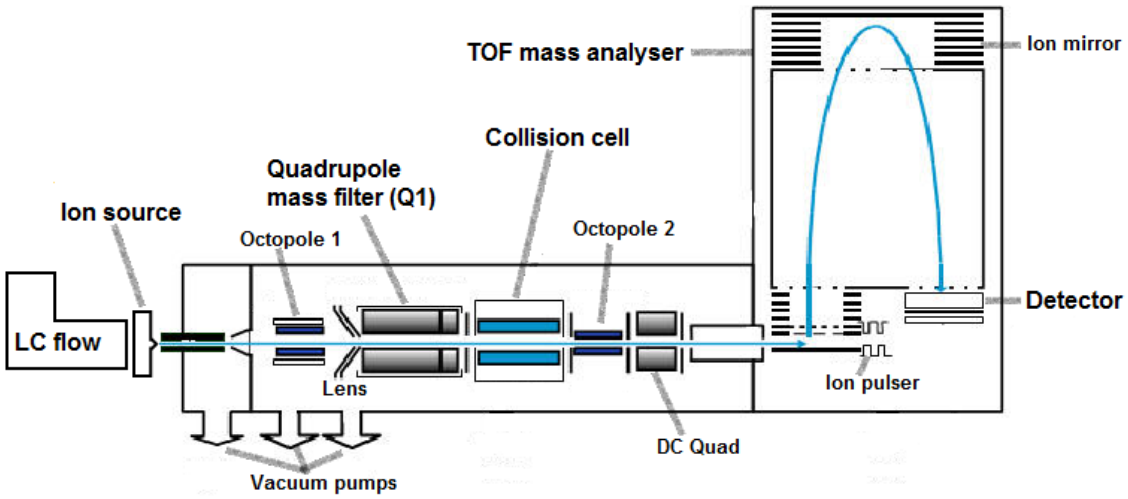


Figure 3.6 A schematic of an LC-QTOF-HRMS used for analyses (Agilent, 2010).

Improvement in scan speed and superior selectivity offered by HRMS, has enabled most laboratories to see HRMS sensitivity comparable or superior to the conventional MS/MS approaches (Korfmacher and Ramanathan, 2016). Zhang et al (2009) investigated the quantitation of small molecules using a linear ion trap HRMS (LTQ-Orbitrap) in comparison with a triple quadrupole (API 4000) operated using selected reaction monitoring and reported comparable validation results and quantitative performance for all drug candidates using the two approaches.

The competitive edge of current HRMS instruments is attributed to the selectivity gains that HRMS allows for due to the accurate mass, which can eliminate interference in samples due to matrix effects leading to a huge increase in signal to noise and overall increase of sensitivity. This is further confirmed by (Bateman et al., 2009; Kaufmann et al., 2011; Ramanathan et al., 2011; Dillen et al., 2012; Henry et al., 2012) demonstrating that quantification capabilities of state-of-the-art HRMS instruments were comparable to the dominant QqQ mass analysers regarding sensitivity and analytical throughput in clinical laboratories. In these projects, various matrices and extraction protocols were adopted and HRMS appeared as specific (owing to high resolution (HR) measurement), robust (due to stable mass accuracy (MA)) and quantitative (owing to good dynamic range and level accuracy) as QqQ-MS.

In comparison with HRMS, LC-MS/MS assays may struggle with interference at lower detection limits from matrix effects and may require the use of complex sample preparation and chromatography to isolate these interferences (Li and Tse, 2010). Whereas HRMS has an in-built selectivity advantage due to its precise mass scanning technology. Also, due to the high resolution employed in mass analysers for HRMS, limits of quantification can be increased in cases where there are interfering peaks in the chromatogram. This means an LC run can be employed for the separation and the MS relied upon for isolation of interference detected in clinical samples due to various factors, example diet, disease state and polypharmacy.

Full scan MS also allows all mass spectral information from a sample to be collected offering the potential for the data to be re-interrogated if it is deemed clinically relevant. In addition, since data is collected simultaneously over the selected mass range it is also possible to directly monitor interfering ions in the sample matrix that could produce ion suppression.

Whilst there is much excitement for the use of HRMS to develop bioanalytical assays, there are still limitations to the wider acceptance across the bioanalytical community. HRMS systems are not as robust compared to QqQ instruments (Huang and Weng, 2013). Rago and Negahban (2016), argue that more guidance is need from regulators on the acceptance of bioanalytical assays developed with HRMS. Currently, data from laboratories employing HRMS is limited. Hence more submissions with the HRMS platform are required to provide industry with the confidence that regulators are on board.

Another challenge with the extension of HRMS to clinical studies is the informed consent process. Taking into consideration targeted LC-MS/MS or targeted HRMS experiments where the analytes are predefined, full scan HRMS analysis preserve all the information about the sample. Hence without proper informed consent from the patient or volunteer, the full benefits of full scan HRMS data cannot be realised. In addition, the wealth of data generated from large full scan data file acquisition presents challenges with software processing and data storage. Table 3.1 compares

the various analytical techniques that have reportedly been used for DBS analysis in terms of cost, sensitivity, specificity and throughput.

Table 3.1 Compares the strengths and limitations of the analytical techniques for the analysis of dried blood samples collected using microsampling methods.

	Cost	Availability	Sensitivity	Specificity	Throughput
Immunoassay	√√√	√√√			
HPLC	√√	√√	√		
LC-MS	√	√	√√	√	√
LC-MS/MS	√	√	√√√	√√√	√√√
LC-HRMS	√	√	√√√	√√√	√√√

3.5.5.1 Advantages of HRMS in comparison with MS/MS

- HRMS does not require molecular fragmentation for selective detection
- HRMS platforms can record various acquisitions at high resolution with accurate mass determination (measure the mass of the molecular ion to better than 1ppm of the RMM) which enables discrimination of compounds with similar masses.
- HRMS is capable of full scan acquisitions
- HRMS full scan acquisition allows a better overview of the analysed extract, because coeluting compounds, contaminants, adducts and charge state can be monitored which are useful during method development and troubleshooting.
- HRMS full scan acquisition does not need experimental determination of collision energies as is required in selected reaction monitoring (SRM) acquisition.
- HRMS allows target and untargeted analysis to be done in parallel.

3.5.5.2 Disadvantages of HRMS in comparison with MS/MS

- Slow progress in embracing the HRMS technology may be due, in part, to difficulties in replacing an entire fleet of triple quadrupole MS in clinical laboratories
- Cost of HRMS instruments compared to QqQ-MS

- Lack of official guidelines by regulatory authorities
- Problems with system robustness

3.5.5.3 Conclusion

Though evidence suggest that LC-MS/MS is the primary technique used for routine determination of therapeutic drug from DBS where the target analytes are readily predetermined, there is evidence that the selective data-acquisition modes of triple quadrupole mass spectrometers (e.g., precursor ion scan or multiple reaction monitoring) are increasingly being replaced by full-scan or MS/MS experiments on HRMS instruments for quantitative applications. This is because the information gained from high-resolution, accurate mass data often outweighs the benefits of highly selective measurements on low-resolution mass spectrometers. The greatest advantage of high-resolution full scan mass spectrometry for TDM of CVD drugs is that all the mass spectral data from the sample is collected. This allows the data to be revisited at a later date if it becomes clinically essential to the management of the patient's condition. For example, in situations where a patient may be taking other medications in addition to the prescribed drug which the clinician is not aware of. Thus, LC-HRMS was selected as the technique of choice for identification and quantification of the selected cardiovascular drugs in dried blood matrix for the medication adherence studies.

Chapter 4 Bioanalytical method development

This chapter discusses the development of the bioanalytical assay (identified as one of the objectives of the study in Chapter 1, section 1.5) using DBS based LC-HRMS assay for the identification and quantification of candidate cardiovascular drugs selected in Chapter 2, section 2.3 (amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan). The extraction procedure and optimisation of LC and MS method parameters are presented since they are of critical importance because the blood spot presents only about 30 µl of sample in total. The resolution of specific analytical challenges discussed in Chapter 3, section 3.4 encountered in identification and quantification for some target analytes from dried blood matrix are also presented.

4.1 Introduction

An objective of the study was to investigate if the presence of specified target drugs could be quantified from small volume dried blood samples collected using DBS cards and volumetric absorptive microsampling (VAMS). Due to the small sample volume, analyses require a very sensitive detection technique. Hence the advantages of the HRMS platform as discussed in Chapter 3, subsection 3.5.5, made it the technique of choice for the determination of the selected candidate cardiovascular drugs in DBS and VAMS sample.

4.1.1 Investigation of punch size

For quantitative analysis of DBS samples, a disk must be punched from the centre of the dried drop of blood. The size of the punch used for cutting the fixed sized disk prior to solvent extraction was investigated using 3mm, 6mm and 8mm diameter punch. A small spot size will produce a small sample. This will affect the amount of analyte extracted from DBS card into solvent. For the 6mm and 8mm punch sizes, the area of the various punch sizes was calculated. The following equation (4.1) was used;

Area $(A) = \pi r^2$ Equation 4.1

Where A is the area, π is a constant and r is the radius of the disk.

It was observed that using a 6mm disk punch would result in wasting more than 50% of the deposited sample on the card. The diameter of a 30µl spot was 9.5mm, with a surface area of 70.88mm^2. The areas of a 6mm and 8mm spot size were 28.27mm^2 and 50.26mm^2 respectively. Owing to the fact that, the difference in surface area between the 6mm and 8mm disk was also more than 30%, the 8mm disk size was maintained for the assay since a bigger area will lead to more sample being taken, which will intend lead to larger detector response with better sensitivities for all analytes. The problem with extraction and low detection limits may be further affected by extraneous material (matrix effect) extracted from the substrate of the card.

4.2 Preliminary method used for the identification of CVD drugs in solution

The starting point for method development was the use of a previously developed bioanalytical method by Drs Tanna and Lawson at De Montfort University (Lawson et al., 2013). This method was used for the quantification of three drugs (bisoprolol, ramipril and simvastatin) in a DBS feasibility study with poor sensitivity reported for simvastatin. This method was used as the foundation for initial trials, improved, built upon and expanded for the analysis of the eleven cardiovascular drugs in small volume blood samples collected using microsampling methods.

Preliminary method instrumental conditions

The chromatographic system used consisted of an Agilent 1290 LC which was coupled to an Agilent G6530A QTOF mass spectrometer, used in the TOF mode. The eleven target drugs and internal standard were chromatographed on an Zorbax Eclipse C18 rapid resolution HD column (Agilent Technologies, Cheshire, UK, 100 mm x 2.1 mm i.d., 1.8µm particle size) which was preceded by a Security Guard Ultra guard column (Phenomenex, Macclesfield, UK). The column oven temperature was set to 40 ◦ C.

Sample injection volume was 2µl. The mobile phase consisted of water containing 0.2% (v/v) formic acid (eluent A) and acetonitrile containing 0.2% (v/v) formic acid (eluent B) and was delivered at 0.6 ml/min with gradient elution. The mobile phase was initiated

at 5% B and maintained for 0.5 min before increasing to 20% B and then to 95% B by 1.5 min and held until 3.0 min before returning to 5% B. The gradient elution programme was then held for 1.5 min to re-equilibrate the column prior to the next injection.

Operation of the mass spectrometer was in electrospray positive ion mode. The MS source and chamber conditions were as follows: fragmentor voltage: 165 V; skimmer: 65 V; drying gas temperature: 350°C; dry gas flow: 10 l/min; nebuliser: 50.0 psig; sheath gas temperature: 400°C; sheath gas flow: 12 l/min. Mass range: 100–1000 m/z; recording rate: 1 Hz. HRMS lock reference masses: 121.0508 m/z and 922.00979 m/z. MassHunter Workstation Acquisition Software for TOF/Q-TOF version B.04.00 (Agilent Technologies) was used to operate the system and acquire all data and the data processed using Qualitative Analysis B.04.00 and Quantitative Analysis B.04.00 software (Agilent Technologies). The mass extraction window used was 5ppm. Calibration of the TOF mass spectrometer was performed daily before starting the analysis.

4.2.1 Experimental

4.2.1.1 Chemicals and materials

Acetonitrile, methanol and water of LC–MS grade were purchased from Fisher Scientific (Loughborough, UK). Amlodipine besylate salt, atenolol (R-(+), 99%), atenolol d_7, atorvastatin calcium salt, bisoprolol hemifumarate salt, diltiazem hydrochloride, doxazosin mesylate salt, lisinopril, losartan potassium salt, ramipril, simvastatin and valsartan were purchased from Sigma–Aldrich (Poole, UK). Autosampler vials and vial caps were purchased from Agilent Technologies (Cheshire, UK).

4.2.2 Analysis and detection of the selected CVD drugs in methanol using the preliminary method.

Standard stock solutions of each target drug amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin, and valsartan were prepared in methanol at a concentration of 1 mg/ml. An intermediate stock

solution of 10,000ng/ml was prepared from each standard stock. A multicomponent working solution of 100ng/ml was prepared from the intermediate stock solutions by diluting with methanol: water (70:30, v/v) and used for LC-HRMS analysis.

The mass to charge (m/z) ratios of the ionised species for all the target drugs which included ($[M+H]^+$, $[M+Na]^+$, $[M+K]^+$) were calculated based on their molecular formula using the Qualitative Analysis software version 4.00 (Mass calculator) as well as in comparison with the m/z values of the drugs reported in the literature (Chapter 2, Table 2.5). The ionised species with the highest intensity signal was used for the detection of each of the target drugs. Results obtained indicated that the molecular (protonated) ion gave the highest intensity signal for atenolol, atenolol d_7, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril and valsartan (Table 4.1). For amlodipine and simvastatin, the abundance was greater for the sodium adduct ion ($[M+Na]^+$). The sodium adduct ion for amlodipine and simvastatin were stable and gave reproducible response and thus was used for identification and quantitation.

Table 4.1 Target cardiovascular drugs, and the mass to charge (m/z) values of the most intense ion used for identification.

Target drug	Ion with highest response	m/z value
Amlodipine	$(M+Na)^+$	431.1344
Atenolol	$(M+H)^+$	267.1703
Atorvastatin	$(M+H)^+$	559.2610
Bisoprolol	$(M+H)^+$	326.2326
Diltiazem	$(M+H)^+$	415.1686
Doxazosin	$(M+H)^+$	452.1928
Lisinopril	$(M+H)^+$	406.2336
Losartan	$(M+H)^+$	423.1695
Ramipril	$(M+H)^+$	417.2384
Simvastatin	$(M+Na)^+$	441.2611
Valsartan	$(M+H)^+$	436.2343
Atenolol d_7 (IS)	$(M+H)^+$	274.2143

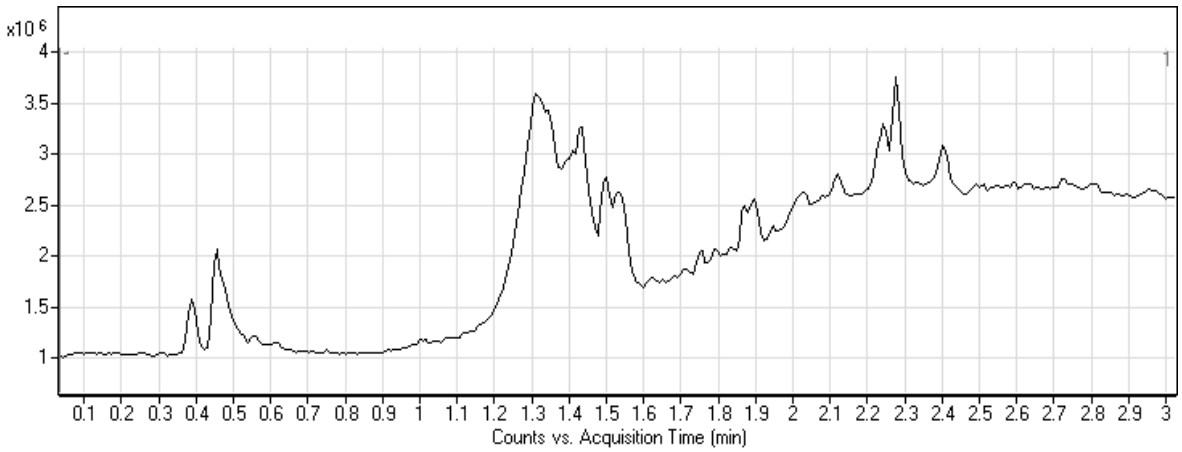


Figure 4.1 Representative LC-HRMS total ion chromatogram (TIC) for a 100ng/ml multicomponent solution of the selected cardiovascular drugs.

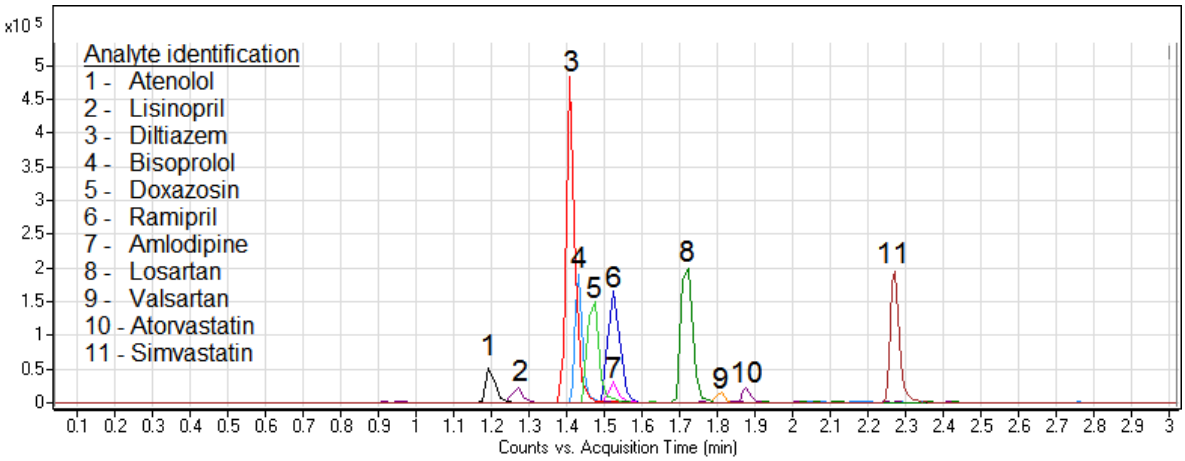


Figure 4.2 Representative LC-HRMS overlaid extracted ion chromatograms (EIC) of a 100ng/ml multicomponent solution standard containing the selected target drugs.

The initial data obtained is the total ion chromatogram (TIC) which records all the ions collected by the TOF during the LC run, this data is shown in Figure 4.1. The presence of each drug is identified by extracting ions of the individual specific m/z value to produce the extracted ion chromatogram (EIC) shown in Figure 4.2.

Though the preliminary method could identify all the cardiovascular drugs, the detector response for amlodipine, atenolol, atorvastatin, lisinopril, and valsartan (Figure 4.2) at the 100ng/ml concentration investigated were relatively low (<50,000 counts) compared to bisoprolol, diltiazem, losartan, ramipril and simvastatin (>150,000 counts). In addition, chromatographic peak shape for amlodipine and lisinopril were

not very sharp. Good peak shape is important for improved chromatographic resolution. As a result, liquid chromatography and mass spectrometry conditions of the preliminary method were optimised for the selected target analytes to improve sensitivity and chromatographic separation for all the target drugs. This was achieved by investigating the LC and MS method parameters such as the LC gradient, amount of mobile phase modifier used, effect of using different analytical columns, the chromatographic run time and post time (Jangala et al., 2014). MS source condition investigated was the fragmentor voltage. The best conditions for optimal responses observed for the selected target drugs were used to create an optimised method.

4.2.3 Preliminary method instrumental optimisation chromatography

The goal of method development was to develop a robust, sensitive, selective and high throughput assay to simultaneously determine the selected CVD drugs in a single run. Due to significant difference in calibration range, the dose strength used in combined therapy as well as physicochemical characteristics such as molecular weight, logP, pKa, polarity, stability and dissolution it was necessary to ensure that the method was suitable for quantitation of all the target drugs. For example, looking at the ionisation constants values of the target drugs: amlodipine (pKa, 8.7), atenolol (pKa, 9.67 and 14.08), atorvastatin (pKa, 4.31), bisoprolol (pKa, 9.67 and 14.09), diltiazem (pKa, 8.18 and 12.86), doxazosin (pKa, 7.24 and 12.67), lisinopril (pKa, 3.17 and 10.21) losartan (pKa, 3.85 and 5.85), ramipril (pKa, 3.75 and 5.20), simvastatin (pKa, 14.91) and valsartan (pKa, 3.9 and 4.7) (Chemicalize, 2017), it is evident that there are significant differences in the degree of ionisation of the compounds. This will affect their extraction from DBS into solvent as the polar compounds will show affinity for polar solvents and non-polar compounds into non-polar solvents. Hence the choice of extraction solvent as well as the extraction procedure is key to successful detection from DBS since no extraction means no detection. It was therefore imperative to suitably optimise the extraction procedure, the liquid chromatography and mass detection conditions, for the subsequent simultaneous determination of the target drugs from DBS. The purpose of optimisation was to ensure that the selected drugs were well resolved on the chromatographic column, with improved MS quantification

and also reduced analyses time. Hence liquid chromatography and mass spectrometry source conditions were investigated to select the best instrumental parameters for the quantification of the selected CVD drugs.

4.2.3.1 Investigation of amount of mobile phase modifier

The pH and ionic strength of the mobile phase can affect the ionisation of the target analytes at the ESI source as well as chromatographic separation. Based on the target analytes, ionic strength and pH can impact peak shape, selectivity and retention (Wagner et al., 2014). However, due to significant differences in the pKa of the selected analytes and since the use of buffer solutions are likely to clog the mass spectrometer, a non-buffered mobile phase was used for separation. Rather formic acid was added to the mobile phase. Ionic modifiers are normally added to the mobile phase to provide a source of protons in LC-MS analysis (Wagner et al., 2014). As a weak acid, formic acid was added to the mobile phase to allow the formation of the desired ions. The use of formic acid in ESI positive mode enhances the ionisation of the positive ions, maintain the ionisation state of analyte in the eluent, improve peak shape and adjust pH of mobile phase (Xu and Madden, 2012; Sargent, 2013). A low mobile phase pH is ideal for acidic analytes to prevent the analytes from being ionised. In addition, most basic (amine) analytes are also suitably retained at low pH due to protonation.

However, the amount of formic acid added to the mobile phase is known to affect the number of ions reaching the detector. Hence the percentage of formic acid was optimised to maintain good peak shapes while being consistent with good ionisation in the mass spectrometer (Nirogi et al., 2006). Since no prior LC-MS work has been reported on most of the selected drugs in DBS, information on previous work from the literature involving simultaneous detection of cardiovascular drugs in plasma and serum was studied (Dias et al., 2013; Kailasam, 2011; Gonzalez et al., 2011; Magiera et al., 2011). In these work, 0.1% formic acid was used as the mobile phase modifier. Investigation was therefore performed with 0.1% formic acid and results compared with using 0.2% formic acid as documented in the preliminary method.

The separation and ionisation of the target drugs were affected by composition of mobile phase. Results indicated that 0.1% formic acid worked best for all the target drugs (Figure 4.3). Peak responses were greater with using 0.1% formic acid in contrast with 0.2% formic acid used as control. The use of 0.1% formic acid showed an increase in peak area response ranging from 8% - 45% for all the target drugs. The results agree with Dias et al (2013) and Gonzalez et al (2011) who used 0.1% formic acid as the amount of mobile phase modifier for the simultaneous quantification of cardiovascular drugs in plasma. This result also indicates that the composition of the mobile phase was a critical factor for achieving good ionisation (Shah et al., 2016). The composition of the mobile phase was therefore optimised as water containing 0.1% formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B).

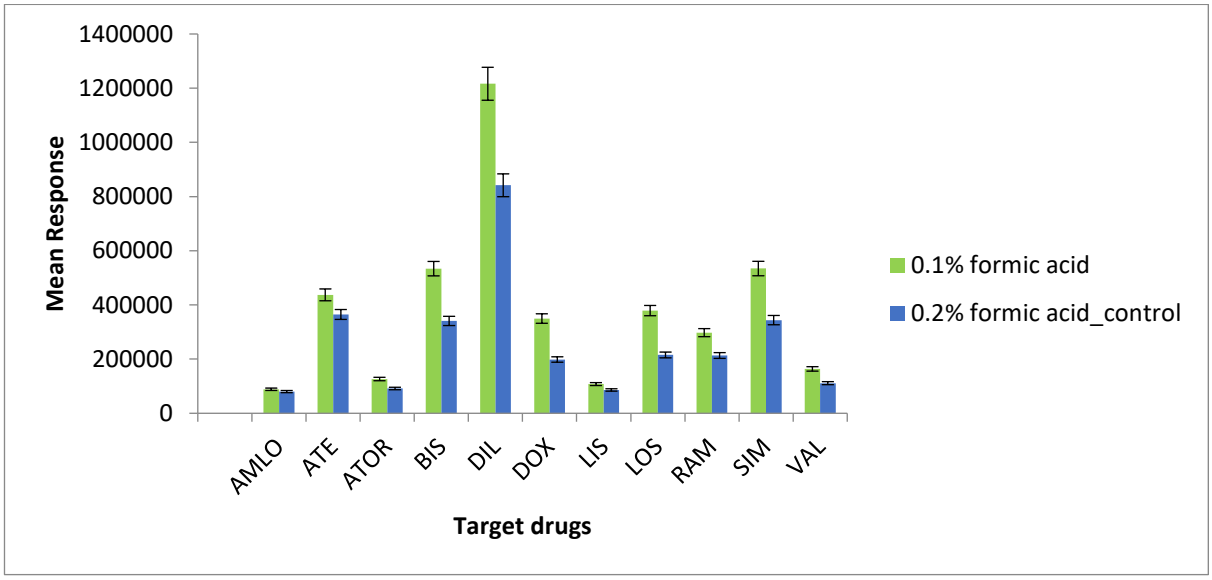


Figure 4.3 A representative plot of mean response for the selected CV drugs using 0.1% and 0.2% formic acid as mobile phase modifier (n = 3).

4.2.3.2 Investigation of gradient time table

In reverse phase chromatography, the separation of a sample with a wide variety of compounds using a single composition of mobile phase will not produce satisfactory retention. For example, when separating compounds that differ widely in polarity, the polar compounds will elute quickly whilst non-polar compounds may be retained on

the column and never elute. Hence using gradient elution which is a process to increase the mobile phase strength as a function of time is always best for separation (Cape et al., 2017). This results in faster analyses, better peak shape and quantitation. LC gradient is therefore a very important parameter to investigate during method development (Snyder et al., 2012). Changing the gradient could impact positively or negatively on chromatographic peak resolution for the target analytes, shorten the run time as well as enhance the MS signal.

Therefore, the gradient timetable under the chromatographic conditions was investigated. Information on the various gradients used in previous studies involving the simultaneous detection of cardiovascular drugs in plasma (Dias et al., 2013; Gonzalez et al., 2011) were obtained from the literature and investigated.

Among the gradients studied, two gradients (B and C) Table 4.2 (Dias et al., 2013; Gonzalez et al., 2011) gave better analyte response compared to the gradient used by Lawson et al (2013) in the preliminary method which served as control.

Table 4.2 Gradient timetable for preliminary method and the two best gradients obtained.

	Gradient A Control (Lawson et al., 2013)			Gradient B (Dias et al., 2013)			Gradient C (Gonzalez et al. 2011)	
	Time/min	B%		Time/min	B%		Time/min	B%
1	0	5	1	0	4	1	0	4
2	0.5	20	2	0.5	40	2	0.5	65
3	1.5	95	3	1.5	98	3	1.5	95
4	3	95	4	3	98	4	3	95
5	3.01	5	5	3.01	4	5	3.01	4

Among the various gradient tables investigated, gradient C (Table 4.2) gave the highest detector response for all the target drugs (Figure 4.4). It was also observed that the target analytes eluted faster with gradient C (Table 4.3), with no compromise to peak resolution allowing the run time to be shortened. This was because of increasing the slope of the gradient by starting the gradient at a higher percent organic. At 0.5 min, the amount of eluent B was 65% for gradient C, compared to 20% and 40% for gradient A and B (Table 4.2). The steepness of the slope caused the analytes to elute faster.

Table 4.3 Comparison of retention times between the three gradients investigated.

Drug	Gradient A_control	Gradient B	Gradient C
	Retention Time (minutes)		
Amlodipine	1.529	1.522	1.005
Atenolol	0.971	0.955	0.811
Atorvastatin	1.866	1.844	1.400
Bisoprolol	1.411	1.404	0.945
Doxazosin	1.478	1.455	0.979
Lisinopril	1.172	1.187	0.862
Losartan	1.711	1.700	1.161
Ramipril	1.512	1.505	1.004
Simvastatin	2.297	2.198	2.000
Valsartan	1.812	1.777	1.300

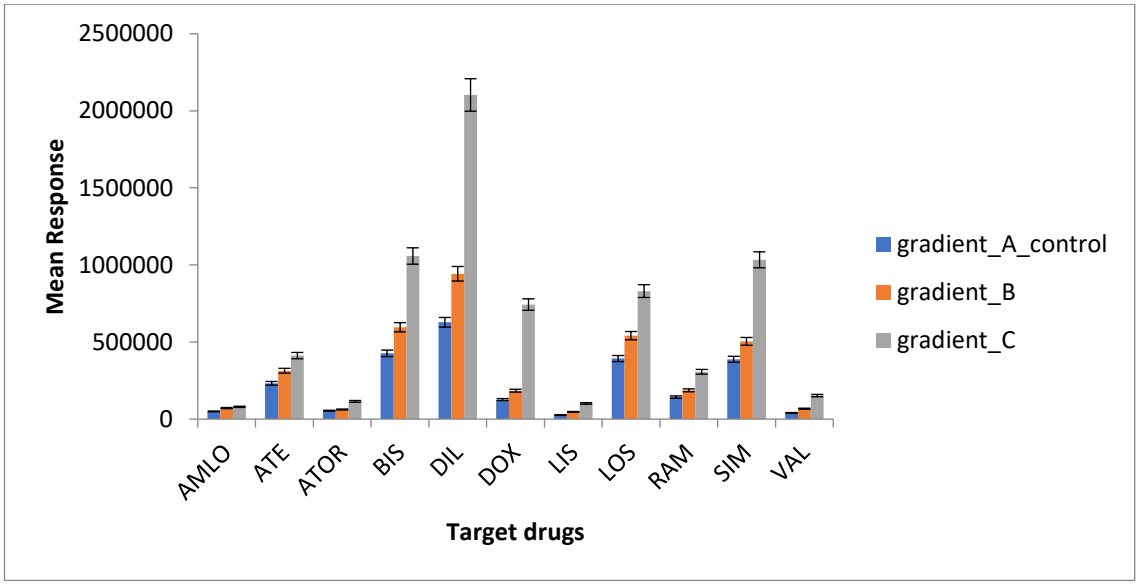


Figure 4.4 Comparison of magnitude of response for each target drug using the three gradients investigated (n = 3).

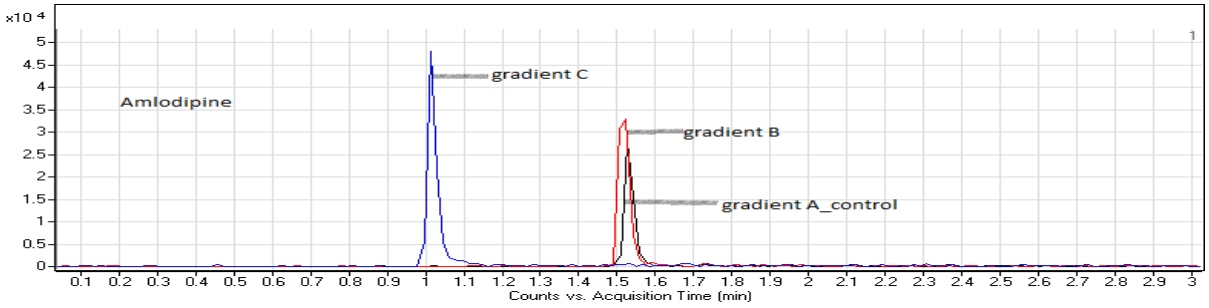


Figure 4.5 Representative EIC for amlodipine with the three gradients investigated

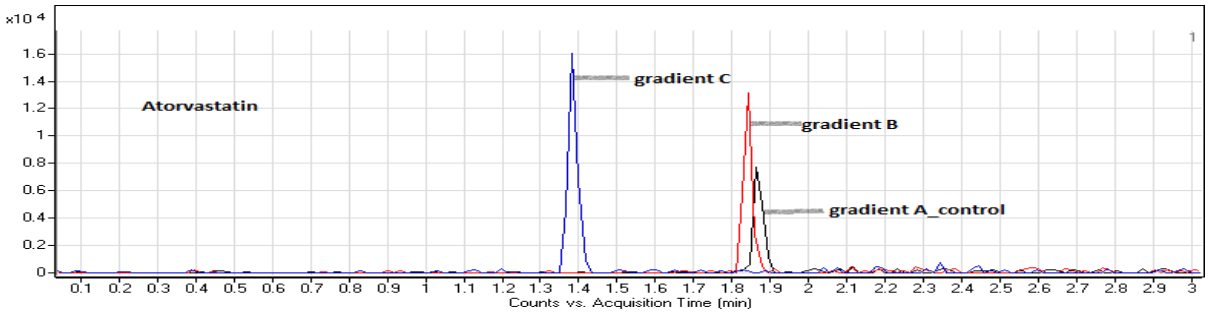


Figure 4.6 Representative EIC for atorvastatin with the three gradients investigated

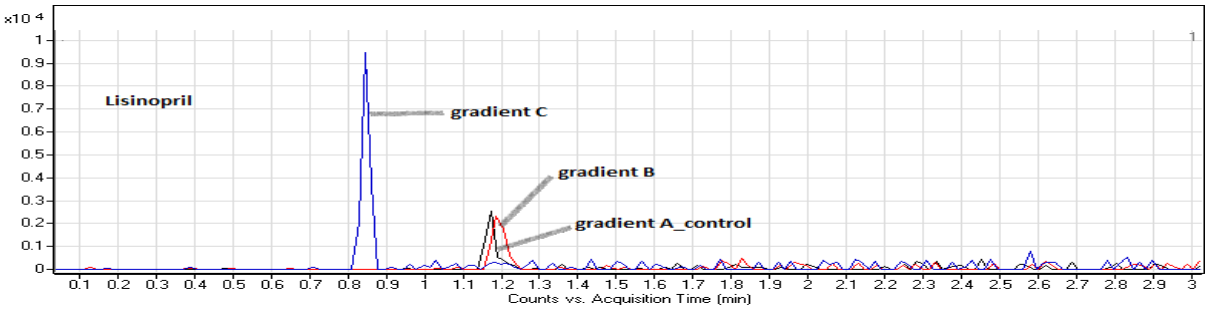


Figure 4.7 Representative EIC for lisinopril with the three gradients investigated

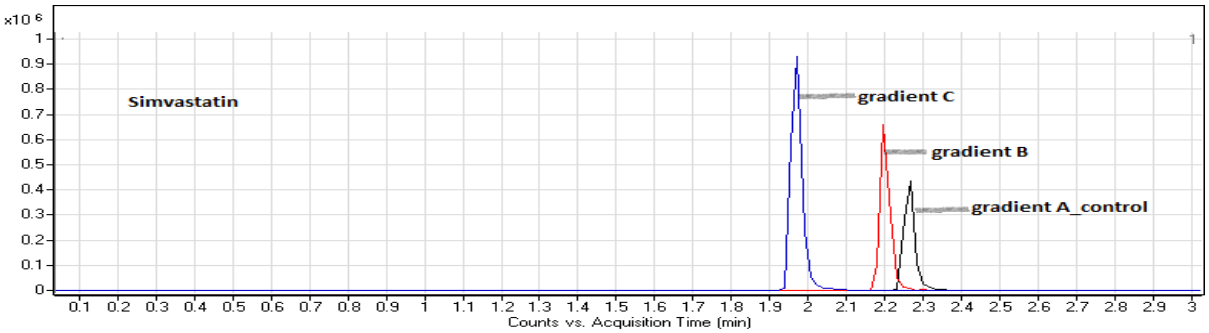


Figure 4.8 Representative EIC for simvastatin with the three gradients investigated

No changes to peak shape were observed for gradients A, B and C. However, there was a significant increase in peak area for all the target analytes (Figure 4.4) when gradient C was compared with gradient A (control). Representative EIC's are shown in (Figure 4.5 - 4.8) for amlodipine, atorvastatin, lisinopril and simvastatin respectively. The increase in response was about 28% for amlodipine and atenolol and greater than 54% for atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril and

simvastatin. Since gradient C gave the highest response for all the target drugs relative to gradient A and B, it was chosen as the new gradient for the optimised method.

4.2.3.3 Investigation of Run Time

Runtime is the time it takes for a complete chromatography cycle. Reducing the chromatographic run time has huge benefits for work efficiency, it increases throughput and saves on cost of expensive solvents used as mobile phases. It also reduces analyses time when the analytical assay is to be used in clinical settings where many samples may be analysed e.g. hospitals. In the preliminary method, the system cycle time was 4.51 minutes consisting of a sample run time of 3.01 minutes and a post time of 1.5 minutes for column re-equilibration. Using the new gradient (gradient C), the chromatographic run time was investigated to look at its effect on magnitude of detector response, peak shape and resolution. Based on the retention time data of the selected target analytes obtained from using gradient C (Table 4.3), runtime was shortened to 2.51 minutes. The mobile phase was initiated at 4% B and held for 0.5 minutes before increasing to 65% B for 1.0 minutes and then to 95% B by 1.5 minutes and maintained until 2.5 minutes before returning to 4% B. Column re-equilibration was achieved by holding the gradient elution programme for 1.5 minutes prior to the next injection.

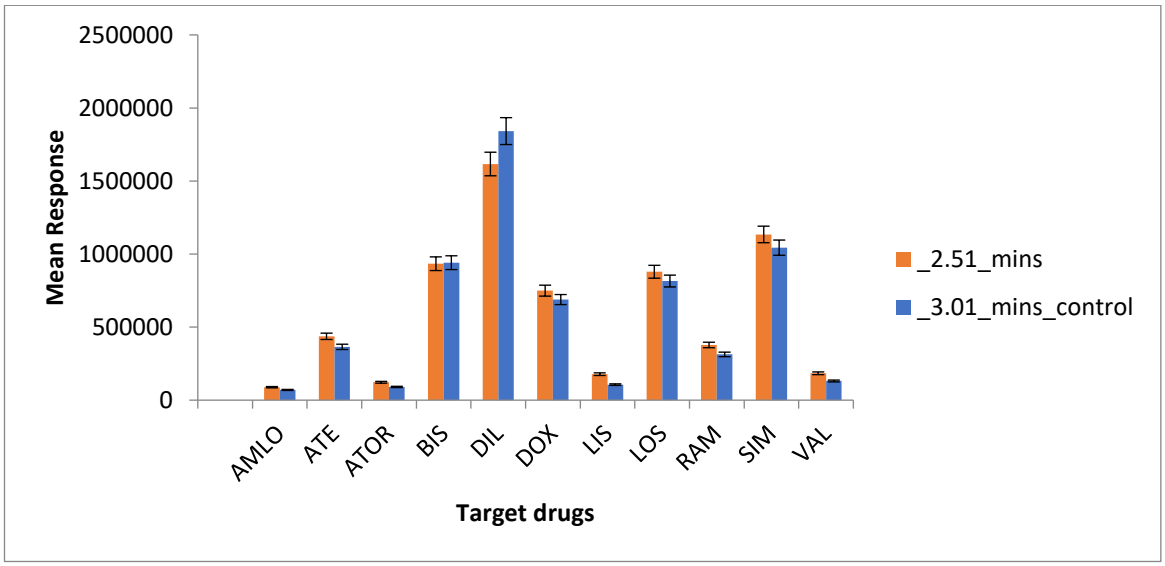


Figure 4.9 Differences in magnitude of response for each target drug using chromatographic run times 2.51 minutes and 3.01 minutes (n = 3).

Shortening the run time from 3.01 minutes to 2.51 minutes impacted positively on the magnitude of response for all the target analytes except diltiazem (Figure 4.9). However, the drop in response for diltiazem was not significant when compared to the gain in response for the other target analytes. ≥ 15% increase in peak area (response) was observed for atenolol, atorvastatin, lisinopril, ramipril, simvastatin and valsartan. ≥ 5% increase was observed for amlodipine, doxazosin and losartan. There were no changes in peak shape and resolution was not affected. Hence the run time was set to 2.51 minutes for the optimised method.

4.2.3.4 Investigation of Post Time

Post time refers to the time it takes to return the analytical column to starting mobile phase conditions after a previous gradient has run. Sufficient column equilibration is necessary between injections so that retention times of the target analytes remain reproducible during method development (Snyder et al., 2012). Changing the post time in a gradient system can affect the time it takes for the back pressure to equilibrate. However, to increase analytical throughput, a method with a short post time is always ideal. Hence the chromatographic post time was changed and investigated by shortening it from 1.5 minutes to 1.0 minutes. Changing the post time should not have any effect on signal size, however the results indicated that, reducing the post time resulted in a drop of response of ≥ 12% for amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, losartan, simvastatin and valsartan (Figure 4.10). This could be due to the fact that the 1.0 minute post time was not sufficient enough to equilibrate the column back pressure before the next injection. Hence the post time of 1.5 minutes was maintained in the optimised method. The results also show good system reproducibility as there were no wide variations in between data collected using a post time of 1.0 and 1.5 minutes respectively.

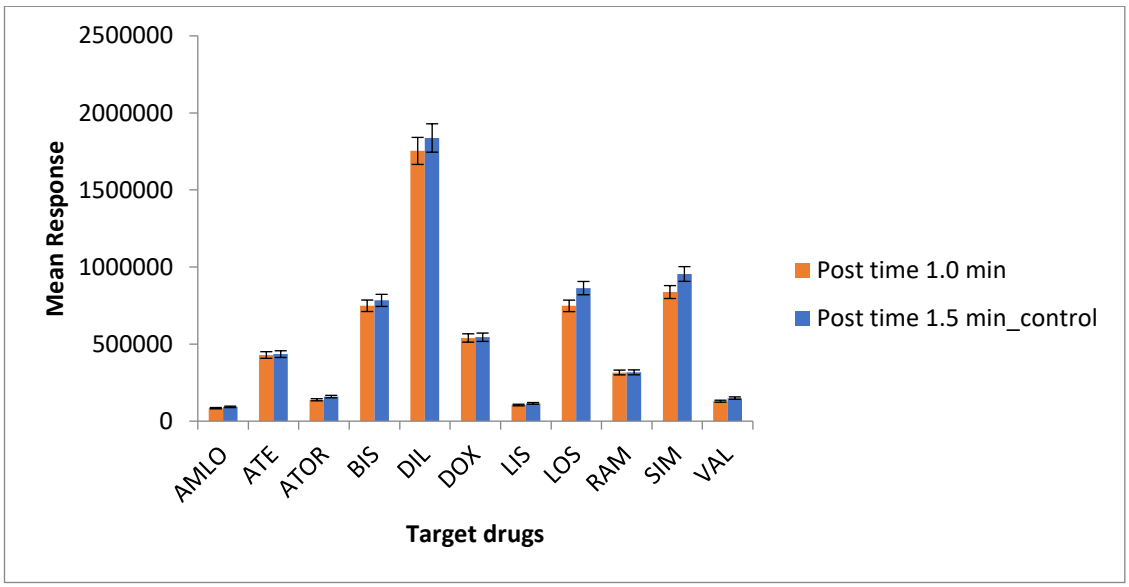


Figure 4.10 Differences in magnitude of response for each target drug using post times 1.0 minutes and 1.5 minutes.

4.2.3.5 New or alternative analytical column development

Finer particle columns are known to give better separation efficiency. Two analytical columns (Zorbax Eclipse C18, 1.8µm, 100mm x 2.1 and Kinetex 1.7µ C18 100 x 2.1mm) were investigated to compare the sensitivity, magnitude of responses and retention times for the selected cardiovascular drugs.

A) A standard stock solution with concentration 1mg/ml was prepared in methanol for each target drug. B) Freshly prepared intermediate solution of concentration 10,000ng/ml was prepared out of the standard stock for each drug. C) Multicomponent working solutions for the concentration ranges 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100, 250ng/ml were prepared for all the target drugs and run on the LC-HRMS using atenolol d_7 as the internal standard. The concentration of internal standard used was 20ng/ml. This concentration was selected on the basis that too high a concentration of the internal standard could suppress the ionisation of the target drugs and reduce sensitivity (Wagner et al., 2014). From previous experiments (section 4.2.2), it was observed that the detector response of atenolol d_7 at the 100ng/ml concentration investigated was about 900,000 counts. However, response for amlodipine, atorvastatin, lisinopril and valsartan were ≤ 200,000 counts, therefore a

concentration of 20ng/ml for atenolol d_7 producing detector response of 200,000 was ideal to prevent ion suppression of the target analytes with low detector response. The analyses were performed in triplicate at each concentration (n = 3).

The calibration curves for the eleven target analytes were generated in replicate (n = 3) using a plot of target analyte/IS peak area ratio against nominal analyte concentration. The magnitude of response, retention times, and LOQ's with a signal to noise ratio of ≥10 for the selected CVD drugs were compared for both columns (Table 4.4).

Table 4.4 Retention times and LOQ's for the target drugs and internal standard on both columns.

Drug	Retention Times		LOQ (ng/ml)	
	Kinetex column	Zorbax column	Kinetex column	Zorbax column
Amlodipine	0.969	1.005	0.1	0.05
Atenolol	0.781	0.828	0.1	0.05
Atorvastatin	1.272	1.400	0.1	0.1
Bisoprolol	0.931	0.965	0.05	0.05
Diltiazem	0.964	1.003	0.05	0.05
Doxazosin	0.935	0.985	0.1	0.1
Lisinopril	0.804	0.862	0.1	0.1
Losartan	1.097	1.168	0.05	0.05
Ramipril	0.981	1.007	0.1	0.1
Simvastatin	1.728	2.005	0.1	0.1
Valsartan	1.213	1.300	0.1	0.1
Atenolol d_7 (IS)	0.784	0.829		

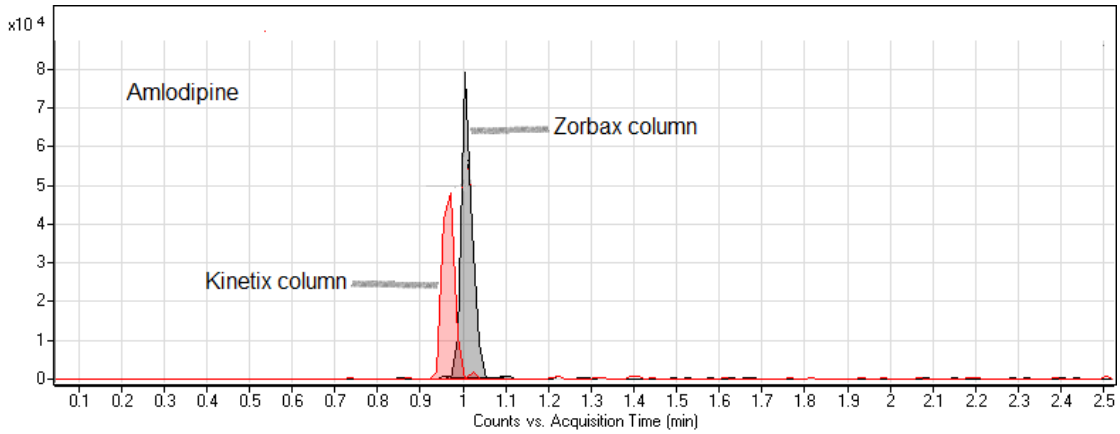


Figure 4.11 Representative EIC of amlodipine at the LOQ on the kinetex and zorbax analytical columns.

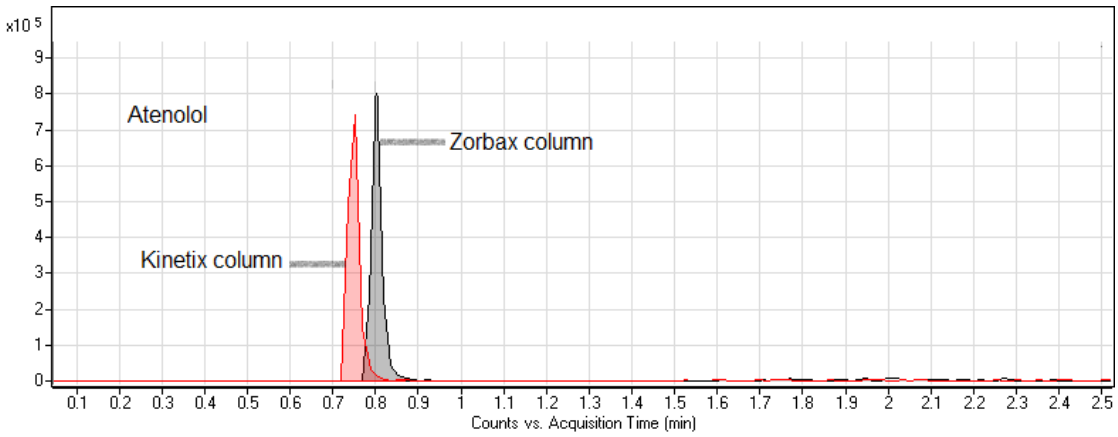


Figure 4.12 Representative EIC of atenolol at the LOQ on the kinetex and zorbax analytical columns.

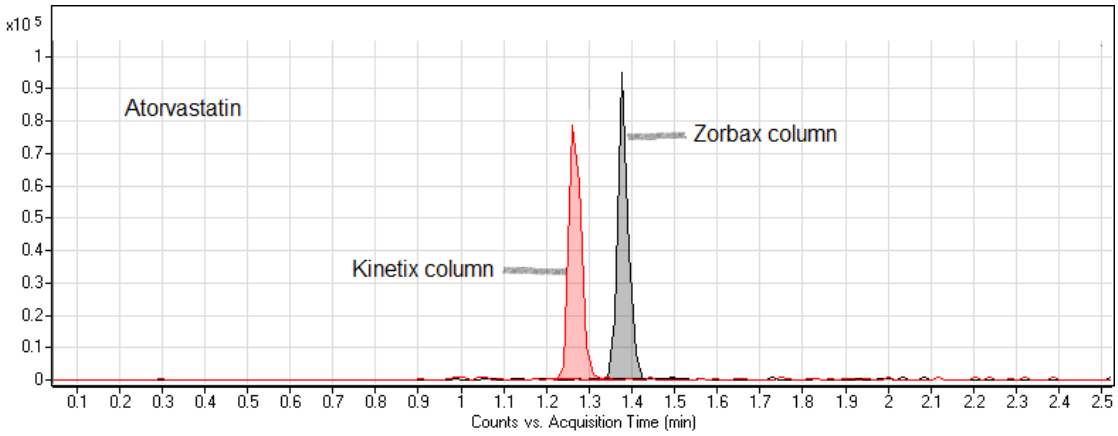


Figure 4.13 Representative EIC of atorvastatin at the LOQ on the kinetex and zorbax analytical columns.

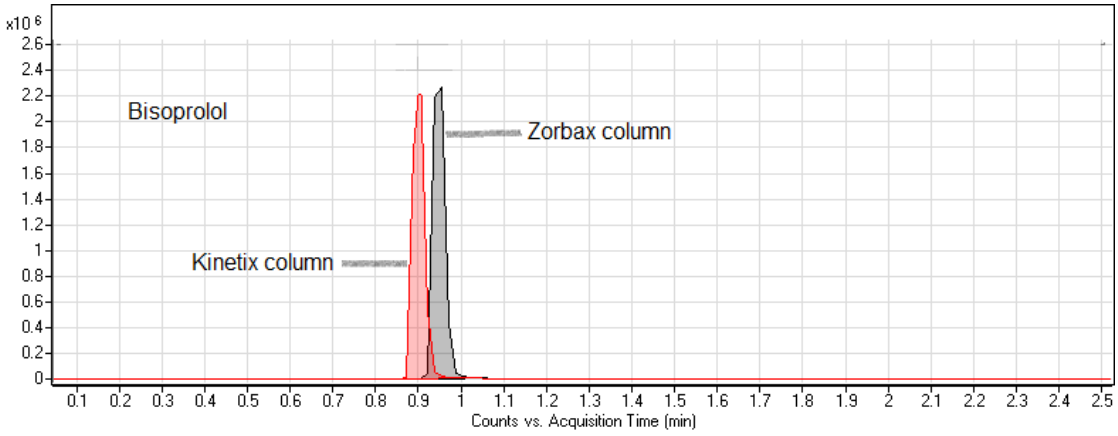


Figure 4.14 Representative EIC of bisoprolol at the LOQ on the kinetex and zorbax analytical columns.

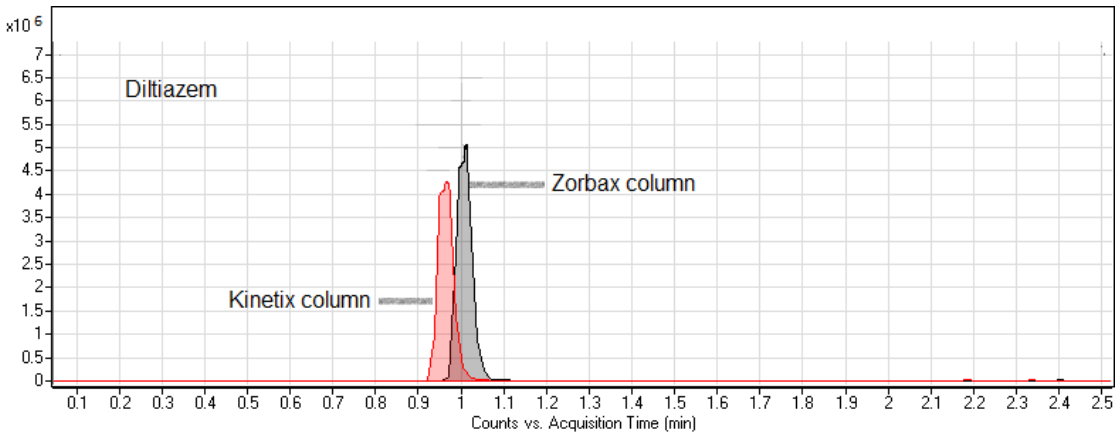


Figure 4.15 Representative EIC of diltiazem at the LOQ on the kinetex and zorbax analytical columns.

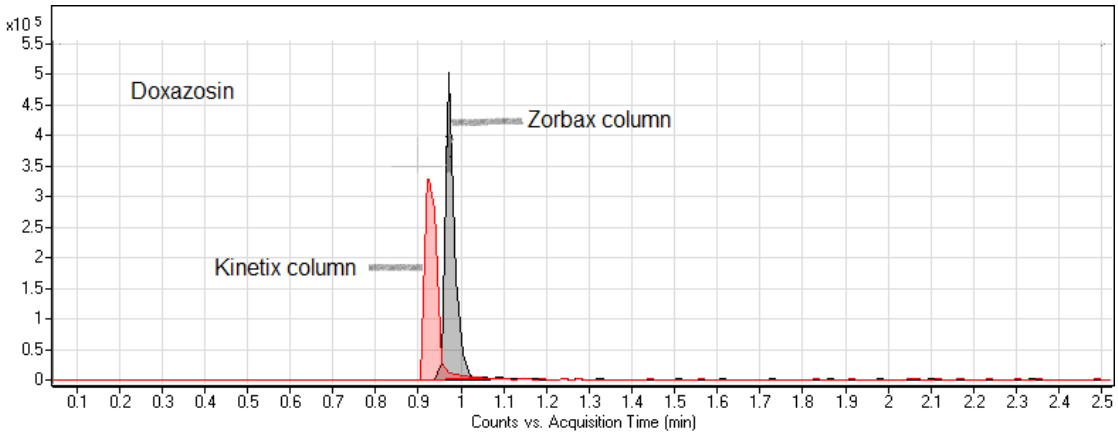


Figure 4.16 Representative EIC of doxazosin at the LOQ on the kinetex and zorbax analytical columns.

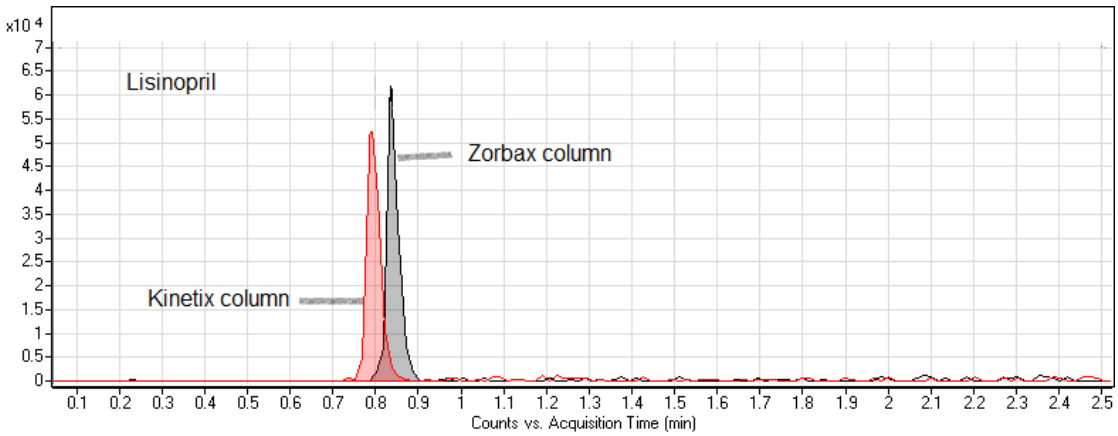


Figure 4.17 Representative EIC of lisinopril at the LOQ on the kinetex and zorbax analytical columns.

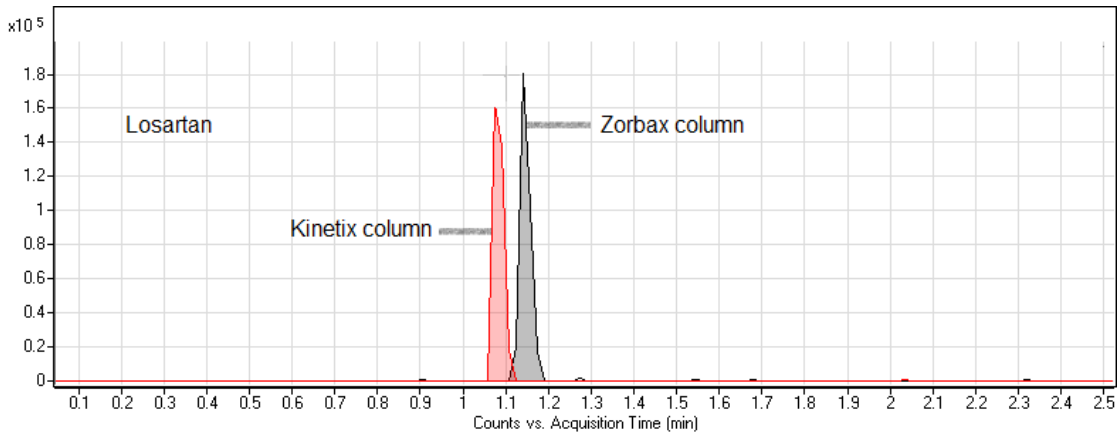


Figure 4.18 Representative EIC of losartan at the LOQ on the kinetex and zorbax analytical columns.

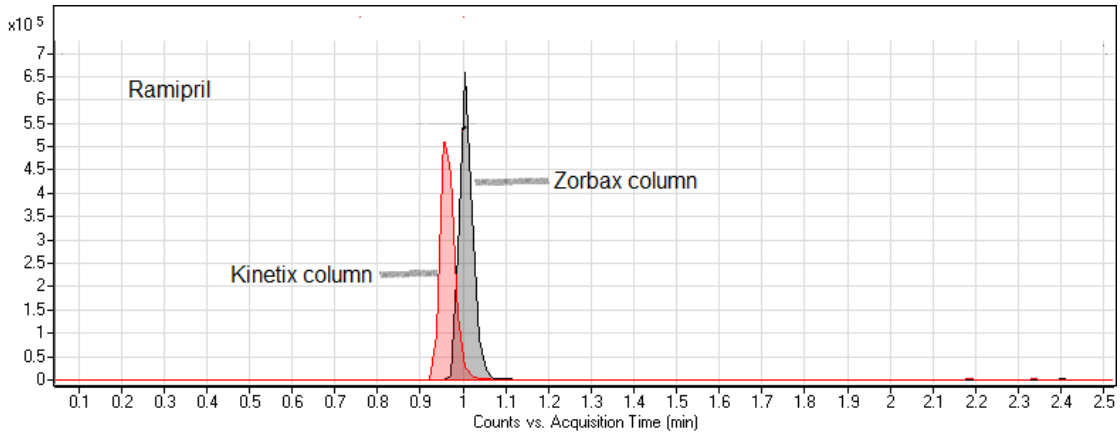


Figure 4.19 Representative EIC of ramipril at the LOQ on the kinetex and zorbax analytical columns.

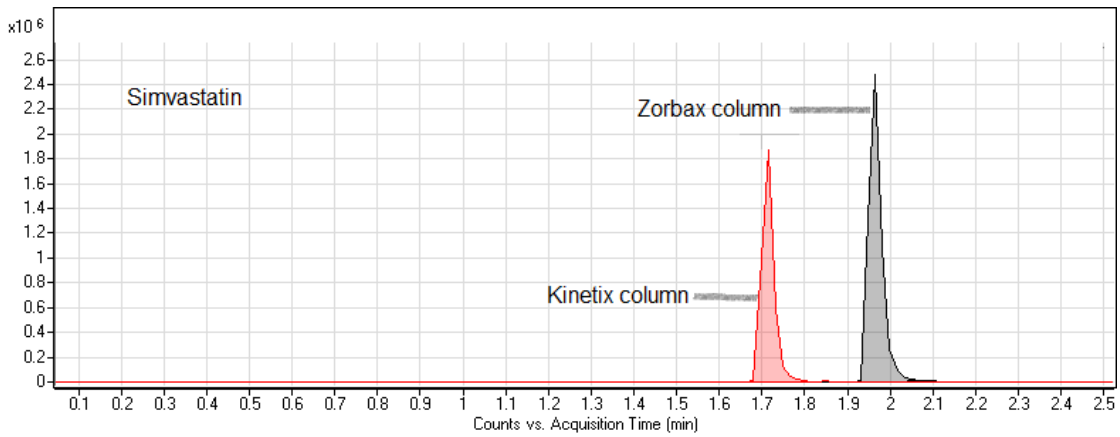


Figure 4.20 Representative EIC of simvastatin at the LOQ on the kinetex and zorbax analytical columns.

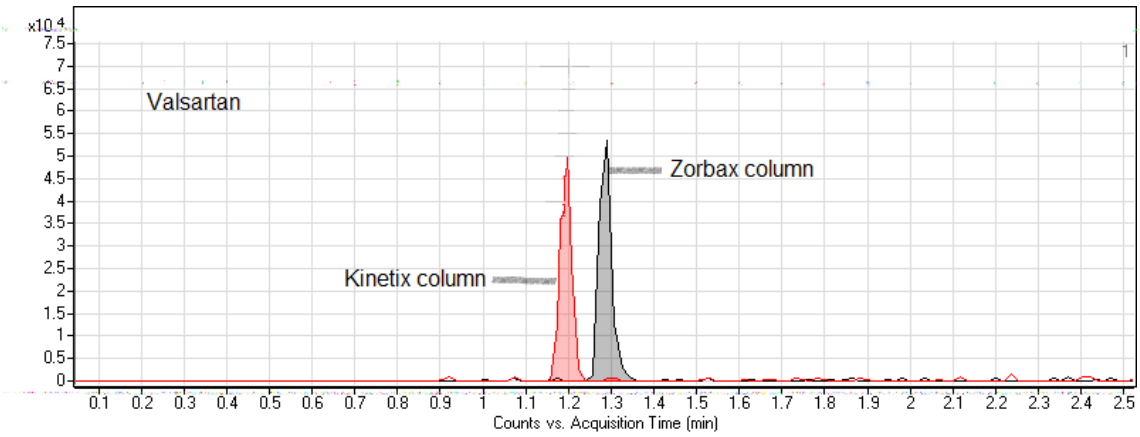


Figure 4.21 Representative EIC of valsartan at the LOQ on the kinetex and zorbax analytical columns.

From Table 4.4, LOQ data are comparable on both columns, for all the target drugs except for amlodipine and atenolol where sensitivities were better on the zorbax column. By convention, smaller particle size columns generally give higher separation efficiencies hence better chromatographic performance (Magiera et al., 2011). Hence, the kinetex column with a particle size of 1.7µ, will be expected to show better sensitivities for the target analytes, but this was not the case. It was also observed that the target analytes eluted faster on the kinetex column (Table 4.4). This is because for small molecules, smaller particle sizes result in fast diffusion, which leads to faster chromatography. Response (peak area) data for both columns indicate that, response on the zorbax column are ≥ 15% high for all the selected CVD drugs, compared to the kinetex column. Representative EIC's are shown for the candidate CVD drugs (Figure 4.11 – 4.21). Hence, the zorbax C18, 2.1 x 100mm, 1.8µm was selected as the column of choice for the developed method.

4.2.4 Method development of mass spectrometry conditions

The MS source condition investigated was the fragmentor voltage for better sensitivity of the selected cardiovascular drugs. Other MS parameters used in the preliminary method were maintained. These include the skimmer: 65 V; drying gas temperature: 350˚C; drying gas flow: 10 l/min; nebuliser: 45.0 psig; sheath gas temperature: 400˚C; sheath gas flow: 12 l/min. Mass range: 100 –1000 m/z; recording rate: 1 Hz.

4.2.4.1 Investigation of fragmentor voltage

For better analyte signal in MS analyses, the MS source and chamber conditions must be optimised for the target analytes being studied. One parameter that significantly affects the ionisation efficiency of target analytes in the ESI chamber is the fragmentor voltage (Rogatsky and Stein, 2005). The fragmentor voltage is particularly important during method development, as it affects the transmission of the ions and also the dissociation of molecules into fragments (Alakhali et al., 2013). Dias et al (2013) reported different fragmentor voltages for different cardiovascular drugs investigated in human plasma. Whilst a significant improvement in detector response (peak area) has been observed for amlodipine, atorvastatin and lisinopril under the optimised LC conditions, peak area response for these drugs were still ≤ 200,000 counts compared to atenolol, bisoprolol, diltiazem, doxazosin, losartan, ramipril, simvastatin and valsartan with an average of ≥ 500,000 counts. Hence under the optimised LC conditions, fragmentor voltage was investigated to determine the best fragmentor voltage for optimised response for amlodipine, atorvastatin and lisinopril and to further improve sensitivity of these three target drugs. The optimum fragmentor voltage is known to be compound dependant and was therefore studied using a 100ng/ml concentration of the CVD drugs at a 2µl injection volume (n=3), between 80 V and 190 V in 5 V increments.

Table 4.5 The target cardiovascular drugs, their mass to charge (m/z) values and the fragmentor voltages with highest analyte signal.

Target drug	Specie with highest response	m/z value	Fragmentor voltage with highest detector response
Amlodipine	$(M+Na)^+$	431.1344	150V/ 170V
Atenolol	$(M+H)^+$	267.1703	140V
Atorvastatin	$(M+H)^+$	559.2610	170V
Bisoprolol	$(M+H)^+$	326.2326	145V
Diltiazem	$(M+H)^+$	415.1686	175V
Doxazosin	$(M+H)^+$	452.1928	140V
Lisinopril	$(M+H)^+$	406.2336	85V/ 150V/ 175V
Losartan	$(M+H)^+$	423.1695	80V
Ramipril	$(M+H)^+$	417.2384	140V
Simvastatin	$(M+Na)^+$	441.2611	85V
Valsartan	$(M+H)^+$	436.2343	85V
Atenolol d_7 (I.S)	$(M+H)^+$	274.1473	140V

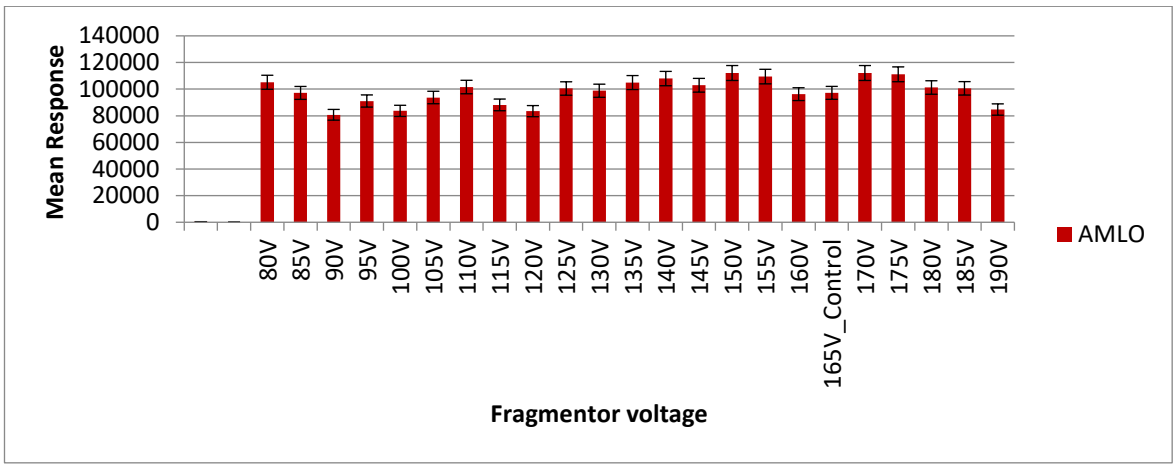


Figure 4.22 A representative plot of mean response against fragmentor voltage for amlodipine (n = 3).

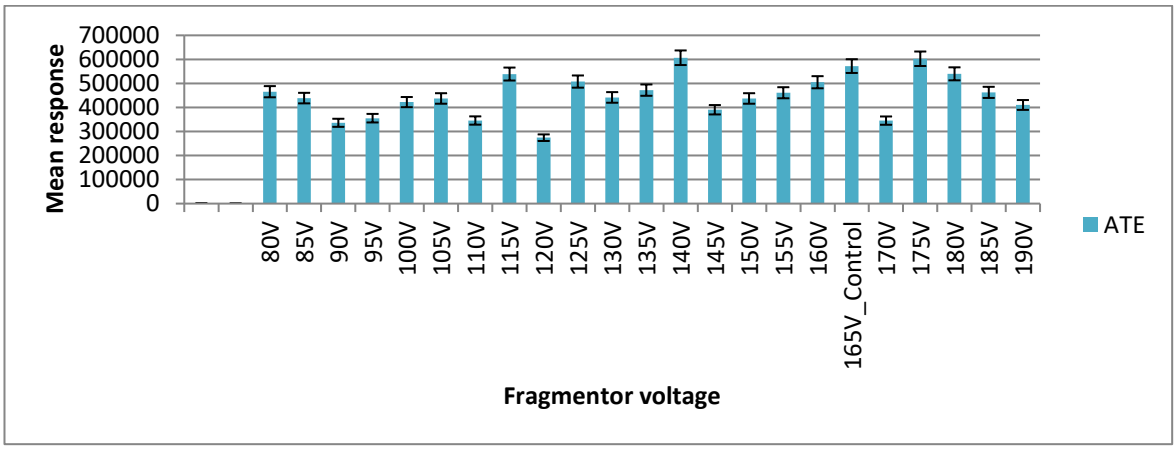


Figure 4.23 A representative plot of mean response against fragmentor voltage for atenolol (n = 3).

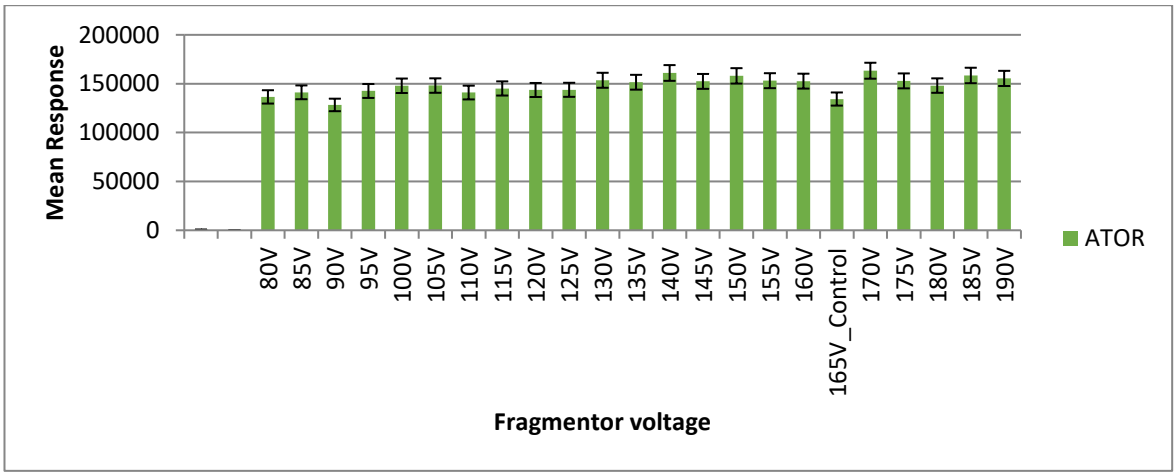


Figure 4.24 A representative plot of mean response against fragmentor voltage for atorvastatin (n = 3).

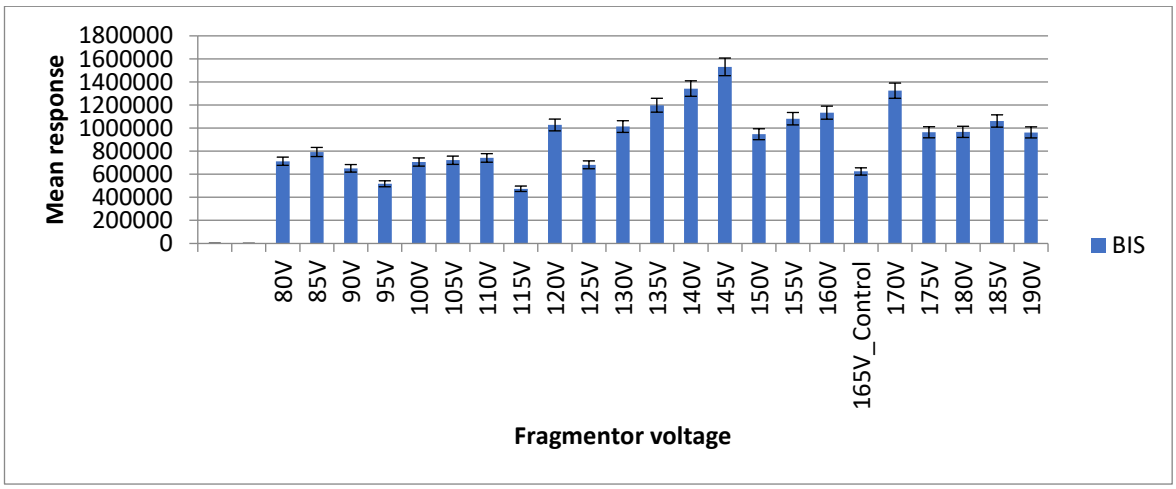


Figure 4.25 A representative plot of mean response against fragmentor voltage for bisoprolol (n = 3).

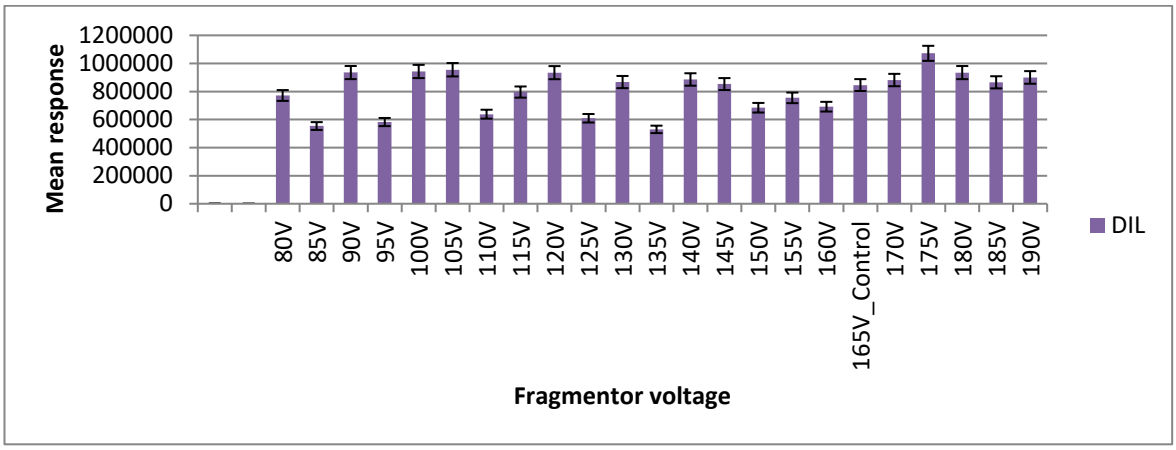


Figure 4.26 A representative plot of mean response against fragmentor voltage for diltiazem (n = 3).

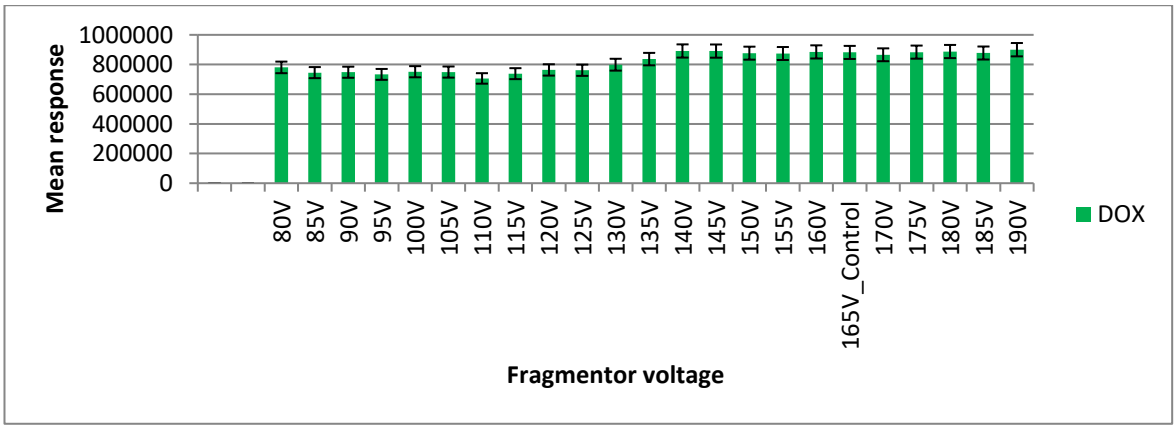


Figure 4.27 A representative plot of mean response against fragmentor voltage for doxazosin (n = 3).

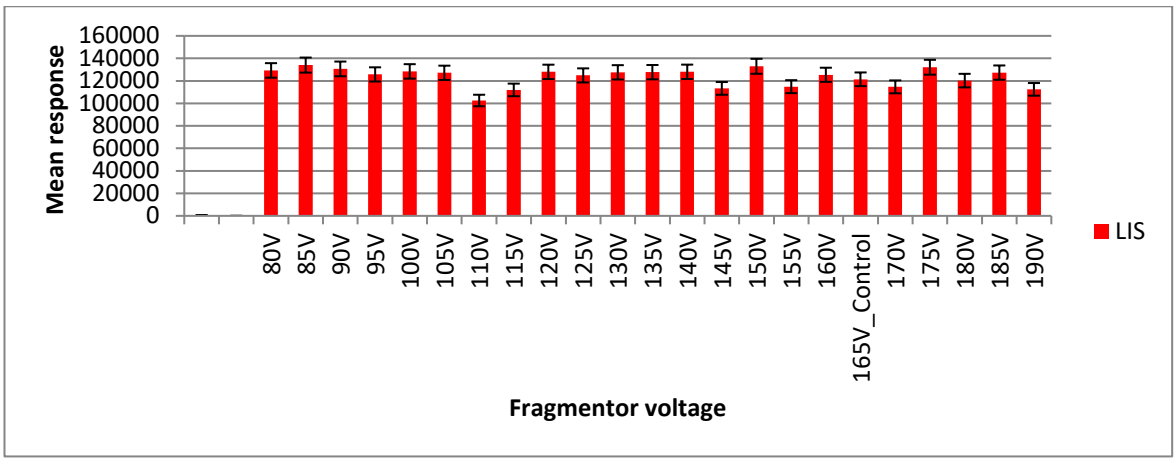


Figure 4.28 A representative plot of mean response against fragmentor voltage for lisinopril (n = 3).

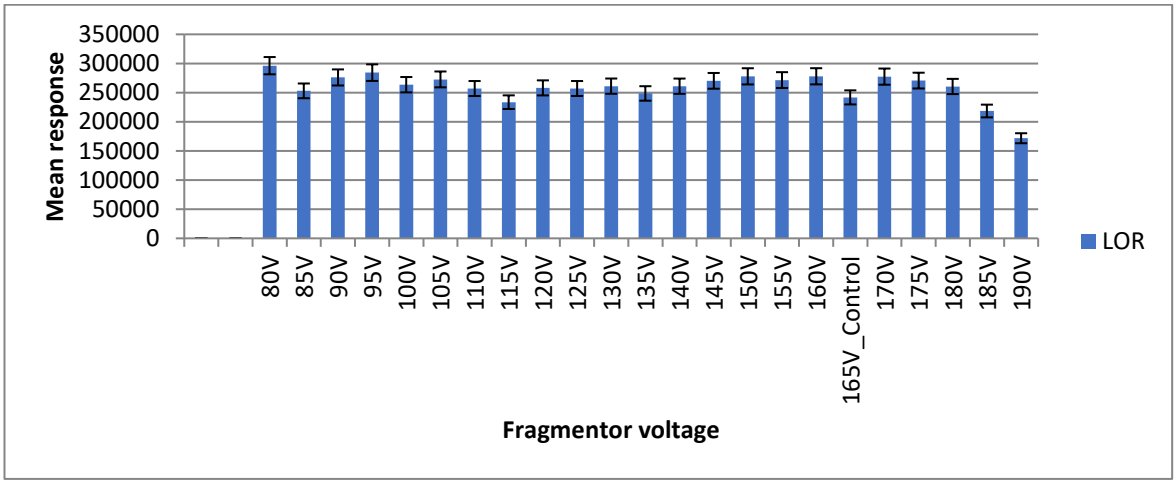


Figure 4.29 A representative plot of mean response against fragmentor voltage for losartan (n = 3).

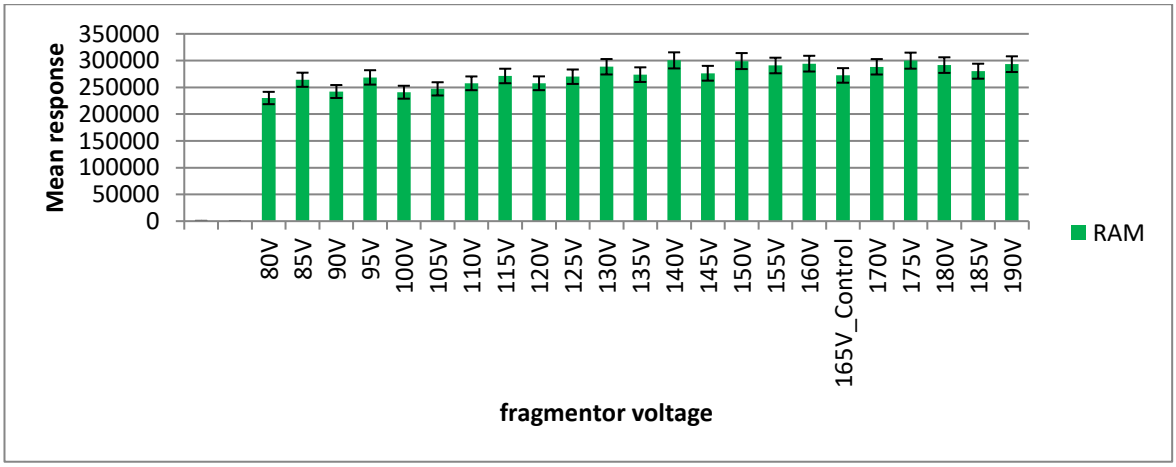


Figure 4.30 A representative plot of mean response against fragmentor voltage for ramipril (n = 3).

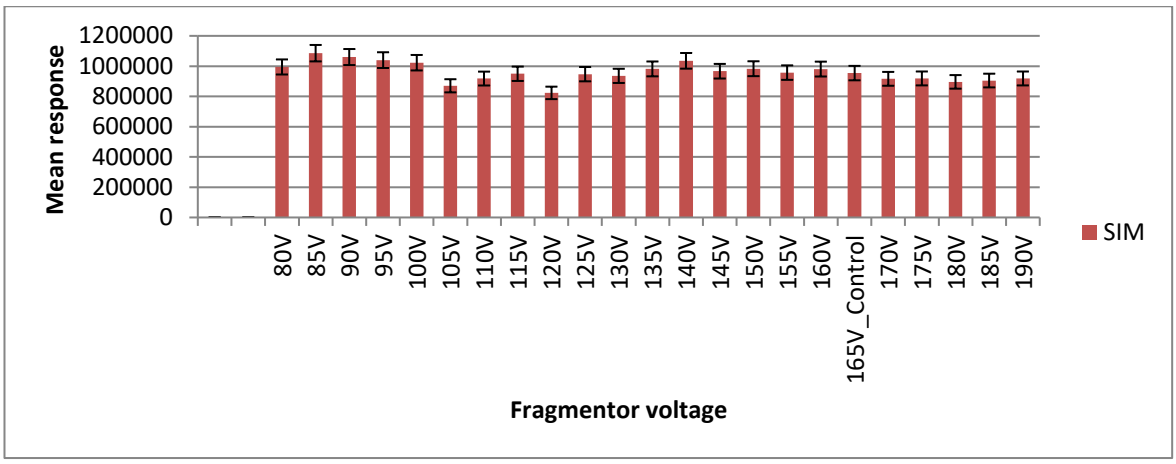


Figure 4.31 A representative plot of mean response against fragmentor voltage for simvastatin (n = 3).

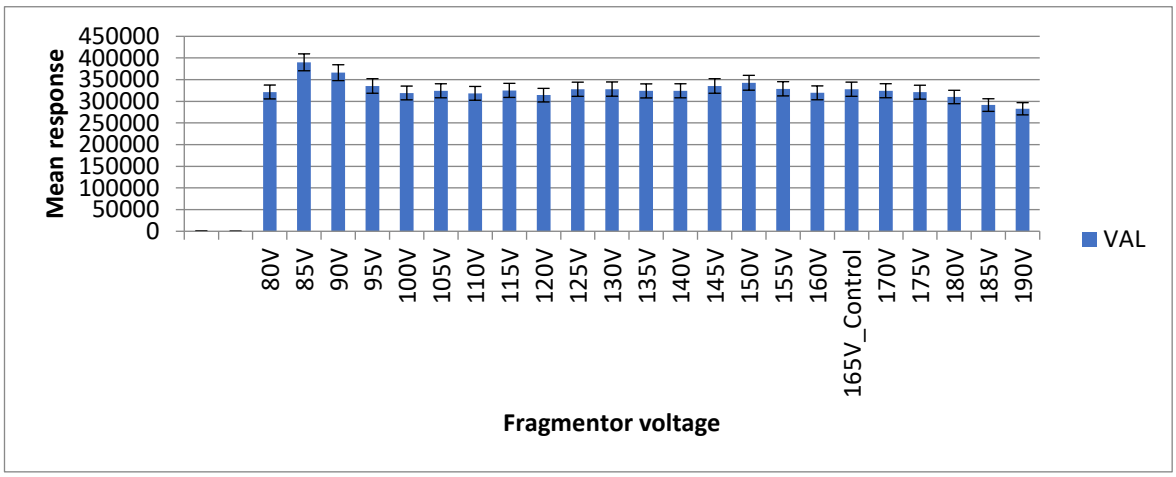


Figure 4.32 A representative plot of mean response against fragmentor voltage for valsartan (n = 3)

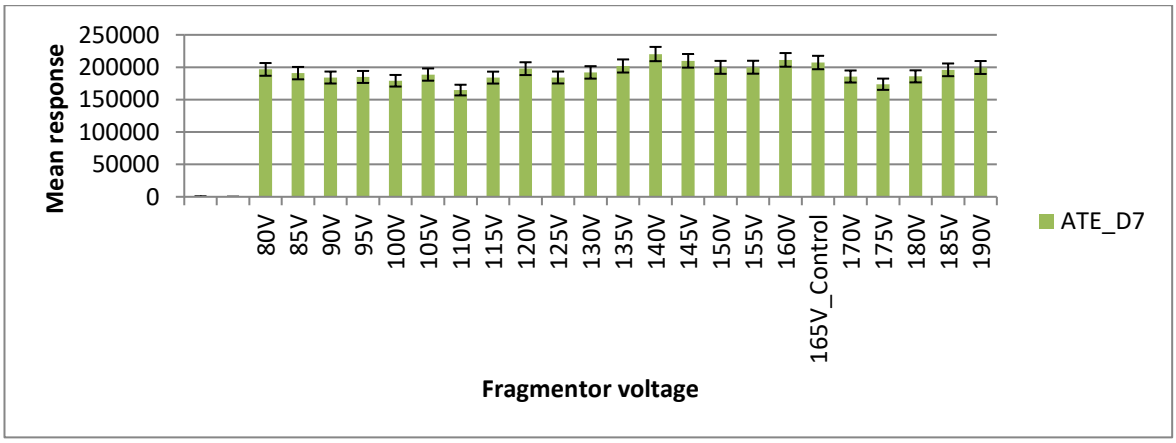


Figure 4.33 A representative plot of mean response against fragmentor voltage for atenolol d_7 (I.S) (n = 3)

Results of the experiment (Table 4.5), confirmed that optimum response for each of the selected targets drugs and internal standard is fragmentor voltage dependent. This result agrees with (Dias et al., 2013) who used different fragmentor voltage for a multianalyte LC-MS/MS analysis of cardiovascular drugs in human plasma. Data from their report indicates that for optimum signal of the targets, fragmentor voltages were different. Since the relative response of amlodipine, atorvastatin and lisinopril compared to the remaining target drugs were ≤ 200,000 counts, the goal was to select a voltage that will boost the response for these drugs. From Figures 4.22, 4.24 and 4.28, the highest responses for amlodipine, atorvastatin, and lisinopril, were obtained at fragmentor voltages 170V, 170V and 85V respectively. However, a fragmentor voltage of 150V was selected as the ideal voltage for the optimised method. This was on the basis that, at 150V, response for amlodipine, atorvastatin and lisinopril were comparable to responses at their optimum fragmentor voltages and response for atenolol, bisoprolol, diltiazem, doxazosin, losartan, ramipril, simvastatin, valsartan and internal standard (atenolol d_7) were not significantly affected (Figure 4.22 – 4.33). Based on the LC and MS parameter investigations, the selected LC and MS conditions used for the optimised method are summarized in Table 4.6.

Table 4.6 A summary of the preliminary and optimised method LC and MS conditions.

	Preliminary method	**Optimised method**
LC conditions	• Mobile phase contains 0.2% formic acid • Mobile phase initiated at 5% B, maintained for 0.5 min, increased to 20% B and then to 95% B by 1.5 min, held until 3.0 min before returning to 5% B. • Run time of 3.01 minutes	• Mobile phase contains 0.1% formic acid • Mobile phase initiated at 4% B, maintained for 0.5 min, increased to 65% B and then to 95% B by 1.5 min, held until 2.5 min before returning to 4% B. • Run time of 2.51 minutes
MS conditions	• Fragmentor voltage: 165 V	• Fragmentor voltage: 150 V

4.3 Application of optimised LC and MS conditions to the analysis of DBS samples

The optimised LC and MS conditions were applied to the analyses of the selected CVD drugs in DBS samples. Target analyte spiked DBS calibration standards were prepared using the standard operating procedure (SOP) developed for the preparation of calibration and validation standards for the 11 target cardiovascular drugs and internal standard in whole blood (Appendix 1). The DBS standard containing the highest concentration of all the target drugs was used for analyses.

4.3.1 Chemicals and materials

Autosampler vials, (250µl) vial inserts and vial caps were purchased from Agilent Technologies (Cheshire, UK). Specimen collection paper type 903, microcentrifuge tubes (1.5 ml), volumetric pipettes, pipette tips and polyethylene bags were obtained from Fisher Scientific (Loughborough, UK). An 8 mm diameter punch was obtained from Maun Industries Ltd. (Nottingham, UK). Lithium heparin coated blood collection tubes were purchased from International Scientifique Supplies Ltd. (Bradford, UK). Fresh blank blood was donated by informed volunteers in line with De Montfort University ethics protocol.

4.3.2 Preparation of calibration and validation DBS samples

Amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan standard and intermediate stock solutions were prepared as detailed in section 4.2.2. Multicomponent working solutions for each target drug were prepared freshly as detailed in the SOP for the preparation of blood calibration standards (Appendix 1).

For the preparation of spiked blood standards, several samples of fresh blank blood (900 µl) were spiked with 100 µl of one of each multicomponent working solution to produce final blood target drug concentrations in Table 4.7. The hematocrit of the blood was 45%. 100 µl of methanol/water (70:30, v/v) was spiked into 900µl of fresh blank blood to produce a zero (blank) blood sample. Internal standard, atenolol d_7

stock solution was prepared in methanol at a concentration of 10µg/ml and diluted further with methanol/water (70:30, v/v) to produce an extraction solvent containing 20 ng/ml of IS. Whilst it is generally recommended to use 5% solvent when preparing DBS calibration and quality control (QC) standards, 10% solvent was used in this assay. Work in this laboratory (Patel, 2011; Lawson et al., 2012) has shown that the use of a 10% solvent standard did not produce any changes to the blood spot spreading.

The calibration ranges were chosen to cover the therapeutic ranges in (Table 2.4 of Chapter 2) for the selected drugs. A minimum of 7-point calibration curve was prepared based on composite data from Chapter 2, Table 2.5 by spotting 30µl of calibration standards including blanks directly onto the 903 sampling paper using a volumetric pipette. The prepared samples were dried at room temperature for at least 3h prior to processing. A 30 µl volume produced a spot of size of ~9.5 mm in diameter on the sampling paper.

Table 4.7 Calibration standards of the 11 target cardiovascular drugs in human whole blood.

Drug	Calibration standards (ng/ml)							
			LOW			MED		HIGH
Amlodipine		0.5	1	5	10	25	50	100
Atenolol	10	20	50	100	200	500	1000	1500
Atorvastatin		0.5	1	5	10	25	50	100
Bisoprolol	0.1	0.5	1	5	10	25	50	100
Diltiazem	0.5	1	5	10	50	100	300	600
Doxazosin	0.1	0.5	1	5	10	25	50	100
Lisinopril	0.1	0.5	1	5	10	25	50	100
Losartan	5	10	25	50	100	250	500	1000
Ramipril	0.1	0.5	1	5	10	25	50	100
Simvastatin	0.1	0.5	1	5	10	25	50	100
Valsartan	50	100	250	500	1000	2000	3000	4000

4.3.3 Extraction of DBS samples

Using the extraction procedure documented in Lawson et al 2013, the analyte spiked DBS standard prepared using the highest concentration of each analyte was solvent extracted for analysis. An 8mm disc was punched from the centre of a 30µl DBS sample and transferred into a clean 1.5ml microcentrifuge tube. 150µl volume of extraction

solvent consisting of MeOH: H_2O (70:30, v/v) was added. The tube was vortexed for 1min, sonicated in an ultrasonic water bath for 30 minutes and centrifuge at 13200rpm for 10 minutes. The supernatant was transferred into an autosampler vial with 250µl insert for analysis.

4.3.3.1 Extract analysis by liquid chromatography high resolution mass spectrometry

Sample injection volume was 20µl. Analysis was performed using the optimised liquid chromatography and mass spectrometry conditions selected for the optimised method as documented in Table 4.6.

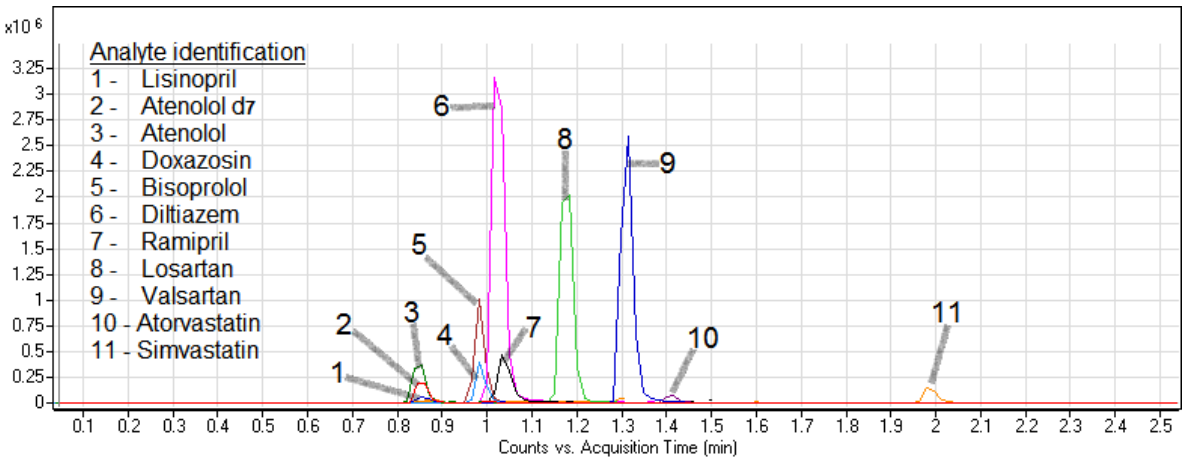


Figure 4.34 Representative LC-HRMS overlaid extracted ion chromatogram of a spiked DBS standard containing the highest concentration of the target drugs and 20ng/ml of IS.

The results obtained indicated that all the target analytes were detectable in spiked DBS standard, except amlodipine (Figure 4.34). Whilst amlodipine was detected and quantified in solvent, it was not detected in DBS when the extraction procedure documented in Lawson et al (2013) was used. In addition, relative detector response for bisoprolol, diltiazem, losartan and valsartan were high, but responses for atenolol, atorvastatin, doxazosin, lisinopril, ramipril and simvastatin in DBS had suffered significantly in DBS matrix (Figure 4.34). Taking doxazosin, ramipril and simvastatin into consideration, initial investigation with a 100ng/ml concentration in solvents did not present any challenges (Figure 4.2), as the compounds were well resolved with good detector response (counts of ≥500,000). However, Figure 4.34 shows that for a

100ng/ml concentration in DBS, the magnitude of responses for these compounds has significantly dropped with counts of (≤300,000).

This was attributed to poor extraction of these target analytes from DBS. The reason was because the card material and blood matrix present challenges for extraction of the analytes. Biological matrices such as blood contain proteins, lipids, salts, cells and many other components likely to interfere with the extraction of the drugs from filter paper. In addition, the selected CVD drugs show significant differences in physicochemical characteristics (molecular weight, logP, logD, polarity, stability, dissolution and thermodynamic properties). Hence, optimising sample extraction was essential to remove most of the unwanted interferents from the DBS extract to make the sample compatible with chromatographic separation and MS analysis.

4.3.4 Optimised extraction of DBS sample

The procedure for analyte extraction from DBS was optimised through trials by investigating parameters such as the extraction solvent, vortexing time, sonication with and without controlled heat, centrifuging time, drying of the supernatant under nitrogen gas and subsequent reconstitution with solvent, addition of formic acid to the reconstitution solvent.

4.3.4.1 Investigation of extraction solvents using (Acetonitrile or Methanol)

Optimal extraction improves the recovery of target analytes from DBS. To optimise the extraction of amlodipine, atenolol, atorvastatin, doxazosin, lisinopril, ramipril and simvastatin in DBS, attention was paid to the extraction procedure documented in previous work, relating to the simultaneous quantification of cardiovascular analytes in plasma and urine (Diaz et al., 2013; Gonzalez et al., 2010; Gonzalez et al., 2011; Tomazewski et al., 2014). It was observed that in these studies, following protein precipitation with organic solvents, the extracts were evaporated to dryness and the filtrate reconstituted in solvents. Hence this procedure was investigated through trials to improve the recovery of amlodipine, atenolol, atorvastatin, doxazosin, lisinopril, ramipril and simvastatin in DBS.

Different volumes (500µl and 300µl) of acetonitrile, methanol and (ACN: MeOH (50:50, v/v)) were investigated for protein precipitation and subsequent extraction of analytes from the DBS sampling paper. The extraction solvent composition documented in Lawson et al (2013) (MeOH: H_2O (70:30, v/v)) was used as control. Following protein precipitation with either acetonitrile, methanol or a mixture of acetonitrile and methanol, the resultant supernatant was removed and dried under a gentle stream of nitrogen gas and the dried residue reconstituted with solvent, vortex mixed for 1 minute and transferred into vials with 250µl inserts for analyses.

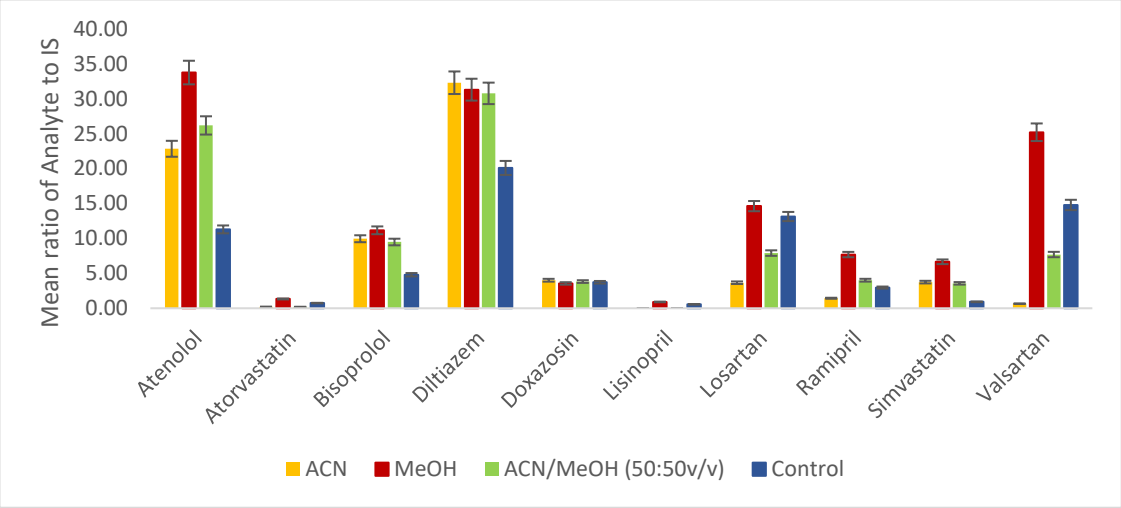


Figure 4.35 Comparison of response in DBS using 300µl of extraction solvents (ACN, MeOH & ACN: MeOH, 50:50, v/v), with 70% MeOH as control (n = 4).

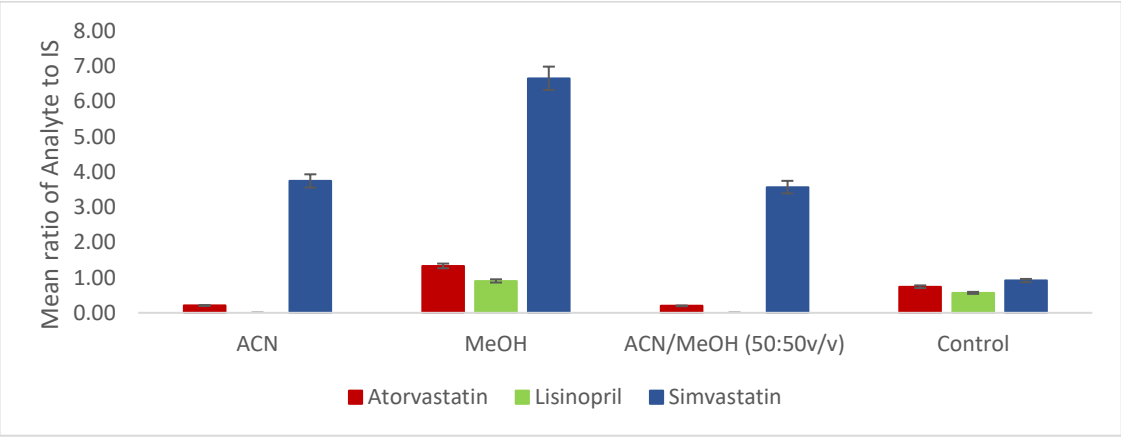


Figure 4.36 Representative comparison of response in DBS using 300µl of extraction solvents (ACN, MeOH & ACN: MeOH, 50:50, v/v), with 70% MeOH as control (n = 4) for atorvastatin, lisinopril and simvastatin.

It was observed that the use of an extraction solvent volume of 500µl was not feasible because it took a long time to evaporate the supernatant to dryness. In view of this the extraction solvent volume used was 300µl. Results obtained indicated that evaporating the supernatant to dryness and reconstitution of the filtrate with solvent significantly improved the recovery of atenolol, atorvastatin, bisoprolol, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan. The gain in detector response ranged from 8% to 72% (Figure 4.35) for the target drugs when compared with the extraction protocol documented in Lawson et al (2013) used as control. For example, atorvastatin, lisinopril and simvastatin which were poorly recovered with the control extraction procedure shows an increase of 54%, 36% and 66% respectively (Figure 4.36). An important observation was that, amlodipine which was initially not detectable with the control extraction procedure, was now detectable using acetonitrile as the extraction solvent. It was however not possible to quantify the peak because the s/n ratio was less than 10.

In contrast with acetonitrile and (MeOH: ACN (50:50, v/v)), it was observed that using methanol as the protein precipitant was the best for the extraction of all the target analytes from DBS except for amlodipine which was not detectable in methanol. Figure 4.35 shows that when acetonitrile is used, there is a slight gain in response (<5%) for diltiazem and doxazosin when compared with methanol. However, lisinopril suffers as it is not quantifiable and there is also significant reduction in detector response for atenolol (33%), atorvastatin (84%), losartan (73%), Ramipril (91%), simvastatin (50%) and valsartan (96%). When a mixture of (MeOH: ACN (50:50, v/v)) was used as the extraction solvent, amlodipine could not be detected and lisinopril was also not quantifiable. Methanol was therefore used for the subsequent extraction of the 10 target analytes in DBS. Since amlodipine could be detected only in acetonitrile, further investigations were performed with acetonitrile as the extraction solvent to optimise the recovery of amlodipine in DBS.

The gains in response observed for atenolol, atorvastatin, bisoprolol, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan could be attributed to the fact that the extracts produced after drying the supernatant and solvent reconstitution

were clean and colourless. Indicating that the filtrate had less matrix interferences when compared with the straw-coloured extract produced using just MeOH: H_2O (70:30, v/v) as extraction solvent (control). This led to a significant reduction in ion suppression at the ESI source of the MS with increased sensitivity.

Investigation of vortexing times, sonication and centrifuging speed and times did not produce any significant gains in recovery of any of the target analytes. The solvent composition used for reconstitution and the volume were also investigated to select the best composition for optimal recovery of the dried extract from the sides of the microcentrifuge tubes.

4.3.4.2 Investigation of reconstitution solvents/volumes using (acetonitrile or methanol) and water

To achieve optimal recovery of the target analytes from the dried extract prior to analysis, a combination of appropriate solvent strength is normally required due their possible differences in solubility (Gonzalez et al., 2010). Hence different ratios of diluent, methanol or acetonitrile with water (Table 4.8) were investigated, to optimise the recovery of analytes from the sides of the microcentrifuge tubes. In addition, the reconstitution solvent volume was also investigated. This was performed by comparing a solvent volume of 150µl and 300µl for dissolving the dried extracts.

Table 4.8 Ratios of (methanol or acetonitrile) with water investigated for reconstitution.

Solvent Composition	Percentages (%)					
Methanol	0	20	40	60	80	100
Water	100	80	60	40	20	0
Acetonitrile	0	20	40	60	80	100
Water	100	80	60	40	20	0

As with the extraction step, acetonitrile worked best for amlodipine and methanol was appropriate for the atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, ramipril, simvastatin and valsartan. Further investigation indicated that MeOH: H_2O (40:60, v/v) solvent composition was the best for the extraction of the ten target analytes from DBS. Whilst ACN: H_2O (40:60, v/v) solvent composition was best for

amlodipine. Figure 4.37 shows representative data for atorvastatin, lisinopril and simvastatin.

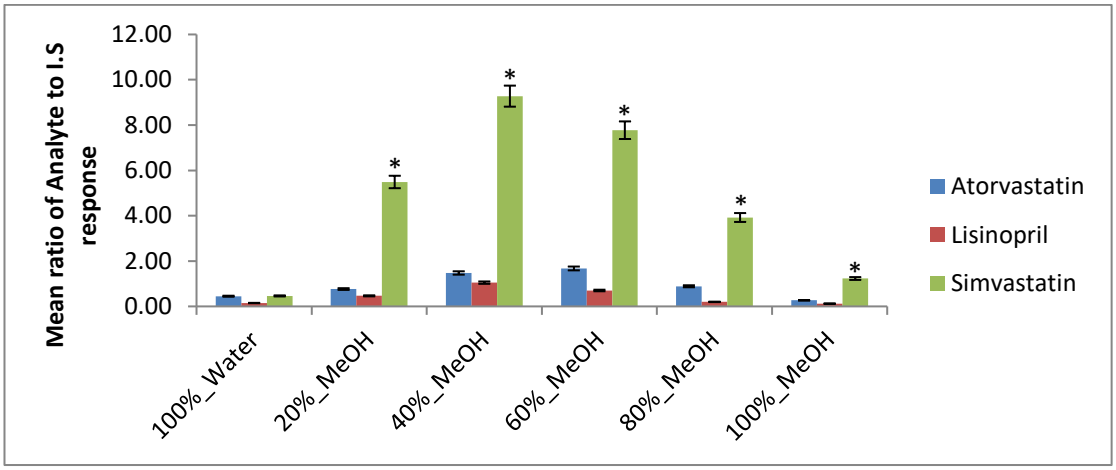


Figure 4.37 Representative plot of reconstitution solvent composition (n = 3) for atorvastatin, lisinopril and simvastatin. For 20%_MeOH, p-value is 0.017; 40%_MeOH, p-value is 0.004; 60%_MeOH, p-value is 0.022; 80% MeOH, p-value is 0.046 and 100%_MeOH p-value is 0.624.

The solvent volume investigation showed that 150µl volume was ideal for reconstitution. Doubling the solvent volume led to a 50% drop in response for all the target analytes. Indicating that the strength of the analytes in solvent is inversely proportional to the volume of solvent used (Strength = 1/volume) and most importantly showing that all the analyte had dissolved in solvent.

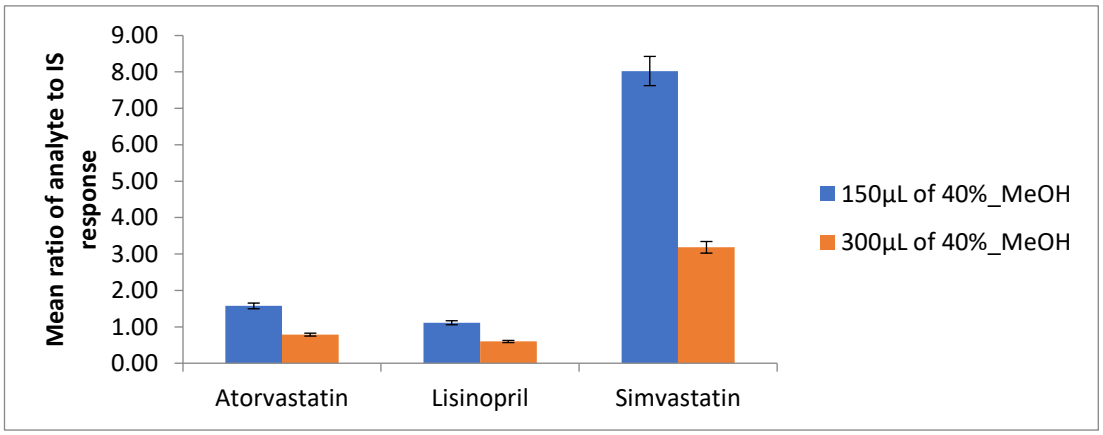


Figure 4.38 Representative plot of reconstitution solvent volume for atorvastatin, lisinopril and simvastatin (n = 4).

4.4 Specific analytical challenges encountered in the quantification of amlodipine, atorvastatin, lisinopril and simvastatin in DBS

The simultaneous quantification of multiple analytes with different physicochemical properties (molecular weight, logP, logD, pKa, polarity, stability, dissolution, thermodynamic properties) and physiological behaviour in dried blood matrix presents challenges due to the following reasons;

- Limited research on dried blood matrix sample pretreatment and extraction procedure for multiple drugs with different physicochemical properties.
- The large differences in concentration ranges of the different analytes in biological fluids. For example, due to the differences in prescribed doses for the selected drugs, the selected ranges investigated were 1 – 100ng/ml for amlodipine, atorvastatin and simvastatin versus 10 – 1500ng/ml for atenolol and 50 – 4000ng/ml for valsartan.

The approach to overcome such challenges was to attempt to develop a robust sample clean up and extraction procedure suitable for all the analytes, but sensitive and selective enough to reduce as much as possible the matrix effects likely to occur in the ESI source of the MS. Among the 11 candidate CVD drugs selected in Chapter 2, section 2.3 for investigation, challenges were encountered with the quantitation of amlodipine, atorvastatin, lisinopril and simvastatin in DBS as reported in section 4.3.3.1. Hence, the following sub section discusses the analytical challenges encountered with the quantification of these drugs in DBS.

4.4.1 Amlodipine

Amlodipine is a compound with polar and non-polar functional groups (Figure 4.39). It is highly soluble in water and has a pKa of 8.7, which means that at physiological pH, it exists primarily in its ionised form (Meredith, 1992). Following oral administration, its bioavailability is 60–65% and plasma concentration rises gradually to peak at 6–8 h after administration.

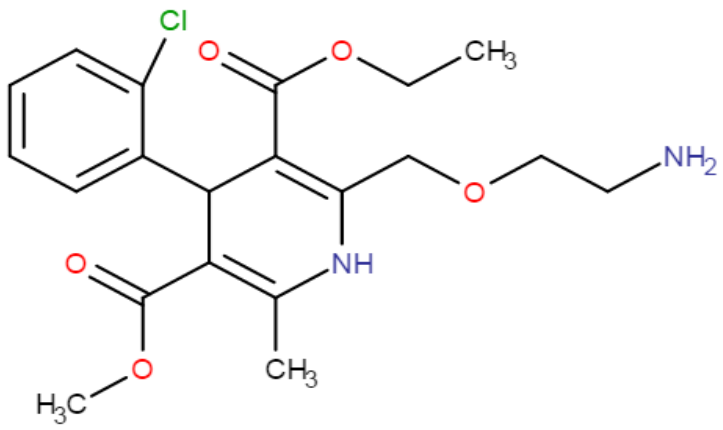


Figure 4.39 Chemical structure of amlodipine.

Amlodipine is reported to have a high degree of protein binding (98%) (Nirogi et al 2006). This explains the reason why amlodipine was not detected in extracts from spiked DBS when the extraction procedure reported in Lawson et al (2013) was used. Precipitation of high molecular weight proteins was performed with methanol: water (70:30, v/v), and since amlodipine is highly bound to plasma protein, it will be retained on the DBS card rather than in the supernatant. Hence hydrolysis of the drug – protein bond will be necessary to free amlodipine and retain it in the extraction solvent. In addition, the 30% ratio of water in the extraction solvent may lead to the dissolution of water soluble components in the blood matrix such as salts likely to cause ion suppression.

Like most other calcium channel antagonist of the dihydropyridine class, a racemic mixture of amlodipine is used for therapeutic purposes (Abernerthy, 1992; Hotha et al., 2013). As a chiral molecule, there is evidence that the opposite enantiomer of a chiral drug often differs significantly in its pharmacological, toxicological, pharmacodynamic and pharmacokinetic properties. (Hotha et al., 2013; Zeng et al., 2010; Mohan et al., 2009; Midha et al., 1998; Islam et al., 1997; Drayer, 1986). Hence the physicochemical properties of both the racemate and the enantiomer, such as polarity, solubility and stability may differ. This leads to differences in solubility and retention time presenting further challenges with detection.

Different extraction techniques (solid phase extraction (SPE) and liquid liquid extraction (LLE)) using different solvents have been reported in the literature for the

extraction of amlodipine from plasma and serum. Shah et al (2017) extracted amlodipine from plasma by solid phase extraction (SPE). 1.0% formic acid was added to plasma samples, before loading unto the cartridge preconditioned with methanol followed by water. Elution of analyte was performed with methanol followed by evaporation of eluate to dryness and finally reconstituting residue in mobile phase solvent. Bathula et al (2012) also extracted amlodipine from plasma using SPE. Plasma samples were loaded unto the cartridge preconditioned with methanol followed by water. Washing was done with water containing 0.1% ammonia and elution was performed with methanol containing 0.1% ammonia.

Conversely, Danafar and Mehrdad (2016) and Chan Mei et al (2013) extracted amlodipine from plasma by liquid–liquid extraction technique using ethyl acetate.

After vortexing and centrifuging the organic layer was transferred to another tube, evaporated to dryness and residue reconstituted with mobile phase solvent. Rezk and Badr (2014) used diethyl ether for the extraction of amlodipine from plasma. After vortexing and centrifuging the organic layer was transferred to another tube, evaporated to dryness and residue reconstituted with mobile phase solvent. In the extraction of amlodipine from plasma, Shentu et al (2012) used acetonitrile followed by the addition of water containing 0.1% formic acid.

Massaroti et al (2005) also extracted amlodipine from plasma using 10µl of NaOH followed by the addition of a mixture of ethyl acetate/hexane (80:20, v/v). After vortexing and centrifuging, the organic layer was transferred to another tube, evaporated to dryness and residue reconstituted with mobile phase solvent. Pandya et al (1995) also extracted amlodipine from plasma samples by incubation for 2 h at 36°C with 2ml of pepsin (proteolytic enzyme) solution to break the drug protein bounds. Plasma protein was then precipitated by the addition of 2ml of 0.2 M borate buffer solution and finally amlodipine was extracted with 2ml of dichloromethane.

The problem of extracting amlodipine from DBS was resolved by using acetonitrile containing 10µl of 0.5M sodium hydroxide (NaOH) and the internal standard as the extraction solvent. Following vortexing, sonication and centrifuging as describe in

Table 4.9, the supernatant was dried down and the dried extracts dissolved with a reconstitution solvent consisting of (acetonitrile: water, 40:60 v/v) with 0.1% formic acid (Chen et al., 2018). The NaOH was added to the extraction solvent (Massaroti et al., 2005; Shentu et al., 2012) to hydrolyse the drug – protein bond. This ensured that amlodipine was retained in solvent, rather than on the DBS card with the precipitated high molecular weight proteins leading to possible detection (Figure 4.40). This procedure resulted in the detection and quantification of the $[M+Na]^+$ ion of amlodipine at m/z 431.1344 (Section 4.2.2, Table 4.1) with a retention time of 1.005 minutes. Methanol could not be used as the extraction solvent for amlodipine because the addition of NaOH produced a supernatant that was dark in colour and could not be used for analyses.

Conversely, all the published papers on the plasma extraction of amlodipine using MS methods for detection used the molecular $[M+H]^+$ ion of amlodipine at m/z 409.2 for quantitation. However, the $[M+H]^+$ ion of amlodipine at m/z 409.1525 with the developed LC-HRMS assay could not be detected in DBS extracts hence the $[M+Na]^+$ ion was used for quantitation.

Table 4.9 Summary of preliminary and optimised extraction procedure.

Preliminary extraction procedure	Optimised extraction procedure
• 8mm disc punched into an eppendorf tube. • 150µl of (70:30v/v) MeOH:H_20 containing 20ng/ml atenolol d_7 as (I.S) added. • Tubes are vortexed for 1 min, sonicated for 30 mins and centrifuged at 13200rpm for 10mins. • 120µl of supernatant transferred into LC vials for analysis.	• 8mm disc punched into an eppendorf tube. • 300µl of (ACN plus 10µl of NaOH) for amlodipine or MeOH containing 20ng/ml atenolol d_7 as (I.S) added for the 10 CVD drugs. • Tubes are vortexed for 1 min, sonicated at 40°C for 30 mins and centrifuged at 13200rpm for 10mins. • 270ul of supernatant are transferred into microcentrifuge tube and dried under a gentle stream of N_2 gas. • Dried residue reconstituted with 150µl of either 40:60v/v MeOH:H_20 or ACN:H_20 containing 0.1% formic acid.

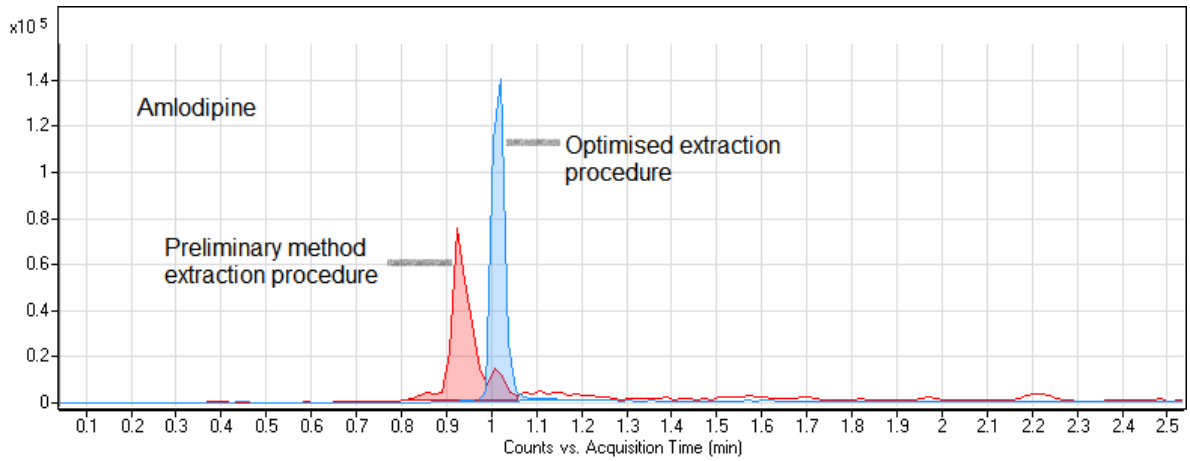


Figure 4.40 Representative LC-HRMS overlaid $[M+Na]^+$ ion EIC's of amlodipine DBS standard using the preliminary and optimised extraction procedures.

4.4.2 Lisinopril

Lisinopril is a hydrophilic molecule containing amino and carboxyl groups (Figure 4.41). After oral administration, the bioavailability of lisinopril is about 25%, but varies widely between individuals (6 to 60%) (Vlase et al., 2010). As a polyfunctional amphoteric compound lisinopril has two basic and two acidic moieties (pKa 10.75, 7.13, 3.13 and 1.63). Thus, lisinopril can produce both negative and positive ions. Previous studies by Qin et al (2007) and Shah et al (2016) have reported that sensitivity for lisinopril was higher in the positive ionisation mode. Being an amphoteric compound, lisinopril is ionised in aqueous solution whatever the pH, thus giving a poor chromatographic peak shape (tailing) on reversed-phase chromatography.

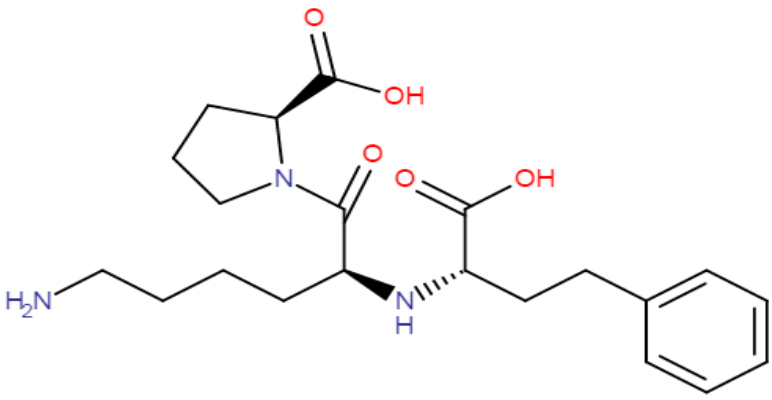


Figure 4.41 Chemical structure of lisinopril.

In addition, lisinopril has been shown to exist as a two (cis and trans) isomer at room temperature (Tsakalof et al., 2002). The rate of isomerization is known to influence peak shape because cis-trans isomers differ in their hydrophobicity. Therefore, lisinopril can produce two separate chromatographic peaks or a good and bad shaped peak depending on column chemistry and mobile phase composition.

Using trifluoroacetic acid (TFA) as additive to the aqueous phase is known to impact positively on the retention of lisinopril and its peak shape on C18 chromatographic column (Vlase et al., 2010). However, the use of TFA in mass spectrometry has a major drawback. The strong ion pair formed by TFA with analyte cannot ionised, thus it will not be detected by the mass spectrometer since ions are required for detection. The poor sensitivity due to TFA can be avoided by using a weaker acid such as formic acid (Vlase et al., 2010). The presence of formic acid in reconstitution solvent improved the peak shape and increased the ionisation of lisinopril (Qin et al., 2011). Using 0.1% formic acid, lisinopril is transformed into a neutral adduct hence the ionisation of carboxylic groups is suppressed by the low pH (Zhou et al., 2008). The formate anion forms a weaker ion-pair with ionised primary and secondary amino groups, neutralizing their charge (Shah et al., 2016). This way the lipophilicity of lisinopril is increased and the chromatographic peak becomes symmetric. Figure 4.42 shows the gains for lisinopril when 0.1% formic acid is added to the reconstitution solvent.

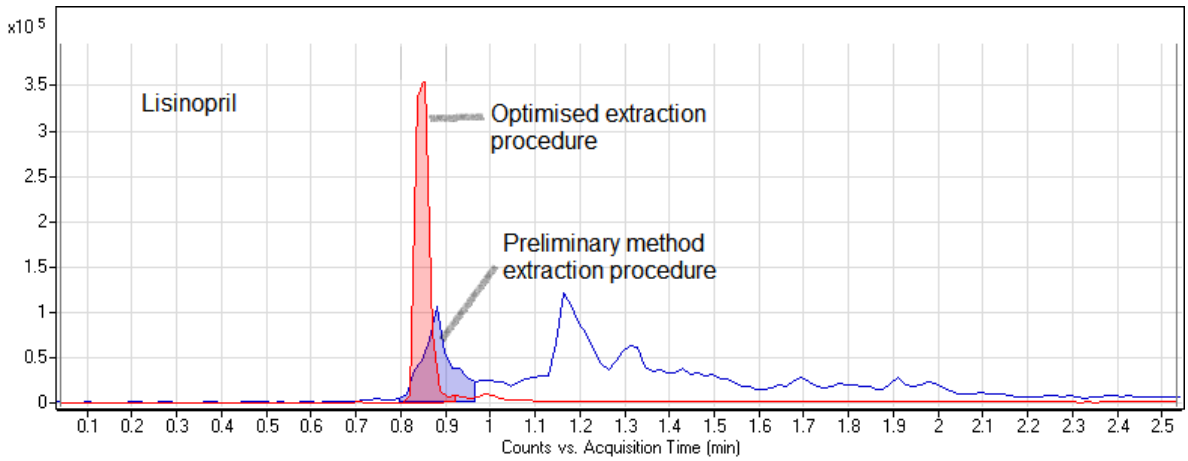


Figure 4.42 Representative LC-HRMS overlaid EIC's of lisinopril DBS standard using the preliminary and optimised extraction procedures.

4.4.3 Atorvastatin and simvastatin

Atorvastatin and simvastatin belong to the group of cardiovascular drugs called statins (Figure 4.43). Statins are lipophilic compounds and their molecules exist in two forms, lactone and open-ring hydroxy acid form (Novakova et al., 2008; Kosek et al., 2017). In vivo, the hydroxy acid forms are the active drugs to lower plasma cholesterol while the lactone forms are inactive prodrug. Lactone form of statins can be absorbed from the gastrointestinal tract and transformed to the active drugs in the liver and non-hepatic tissues.

Atorvastatin is administered in the open-ring hydroxy acid form, the active form and gets completely absorbed upon oral administration (Partini et al., 2013). It has an oral bioavailability of about 12–14% due to its rapid presystemic clearance in the gut wall as well as metabolism in the liver (Kosek et al., 2017). Atorvastatin is also highly bound to plasma protein (~96%) and has the elimination half-life of approximately 14h, which is considerably longer than that of most other statins (Androw, 1997). It is extensively metabolized to its 2- and 4-hydroxylated derivatives and various β-oxidation products (Partini et al., 2013).

Simvastatin on the other hand is a prodrug, which is administered as an inactive lactone. The lactone is absorbed from gastrointestinal tract and hydrolyzed to the active-hydroxy acid form in the liver (Ahmed et al 2012). The bioavailability of simvastatin is very low and reported to be 5% (Patel and Kothari, 2017; Vetrova et al., 2015).

Statins are a typical example of drugs, where there is interconversion between lactone and open-ring hydroxy acid (Jemal and Xia, 2000; Yang et al., 2006). This interconversion can take place during sample preparation and the analyses stage leading to inaccuracies of results. Hence, it is essential to select conditions that will eliminate or minimise their in-vitro interconversion during method development. It's been reported that adjusting pH to between pH 4 and pH 5, significantly reduce the interconversion between lactone and acid forms (Jemal et al., 2000; Ahmed et al., 2012, Partini et al., 2013). Since statins are extreme hydrophobic compounds, they are

known to suffer significant matrix effects during analyses in matrices such as blood or plasma (Dias et al., 2013).

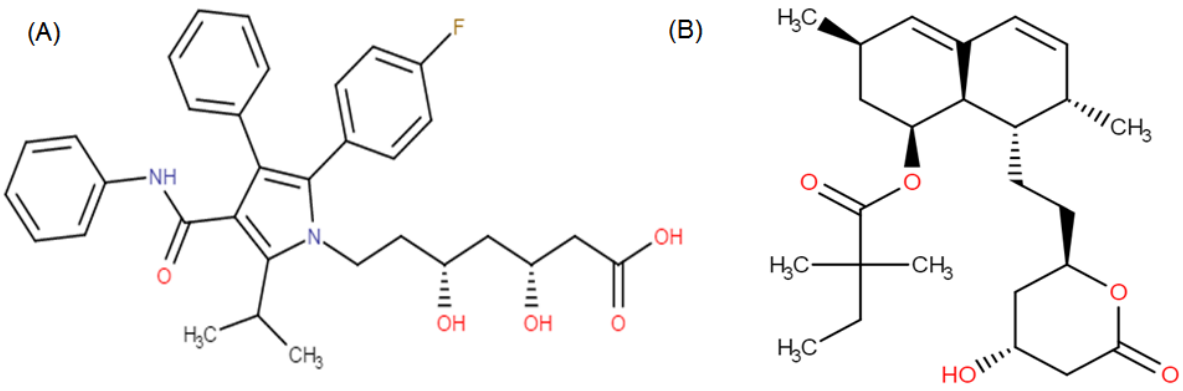


Figure 4.43 Chemical structure of (a) atorvastatin and (b) simvastatin.

To select conditions towards optimising the extraction of the statins, attention was paid to previous work, relating to the estimation of atorvastatin and simvastatin in plasma. Evaporating the supernatant to dryness and subsequent reconstitution significantly reduced the matrix effect and led to improved recovery of both statins due to the clear and colourless filtrates produced. In addition, acidifying the reconstitution solvent with 0.1% formic acid will lower the pH of the extract and hence minimised any possible interconversions of between the lactone and hydroxyl acid forms. Heat enhances solubility, hence application of moderate heat during sonication on a water bath, improved the recovery of atorvastatin and simvastatin. Heating of the water bath during sonication was experimented at 40˚C and 60˚C, with another extraction conducted with no heat serving as control. At 40˚C there were gain of about 18% and 36% for atorvastatin and simvastatin respectively (Figure 4.44). Figure 4.45 and 4.46 shows the improvements made to the recovery of atorvastatin and simvastatin.

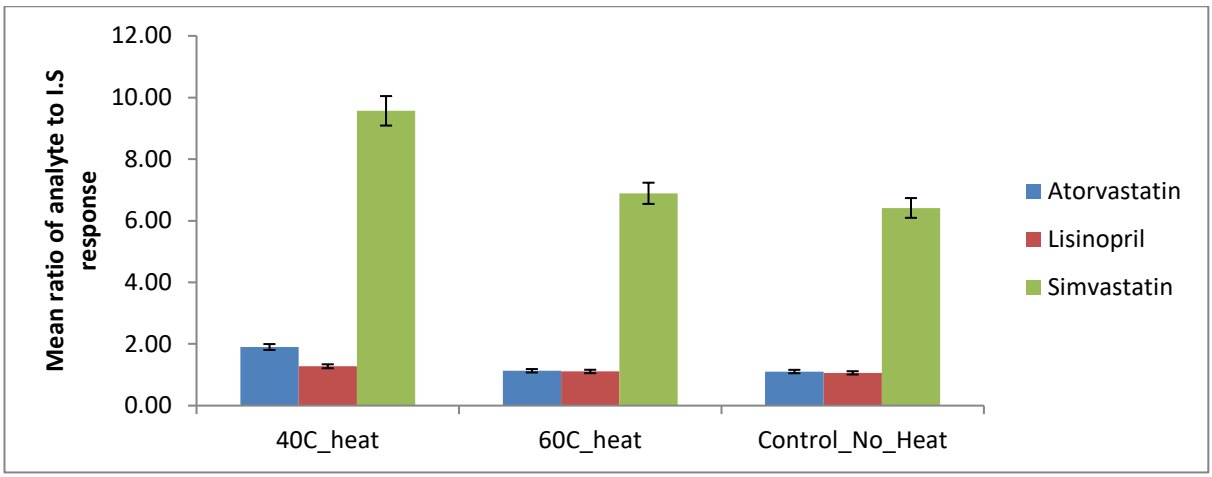


Figure 4.44 Effect of heat on the extraction of atorvastatin, lisinopril and simvastatin (n = 4).

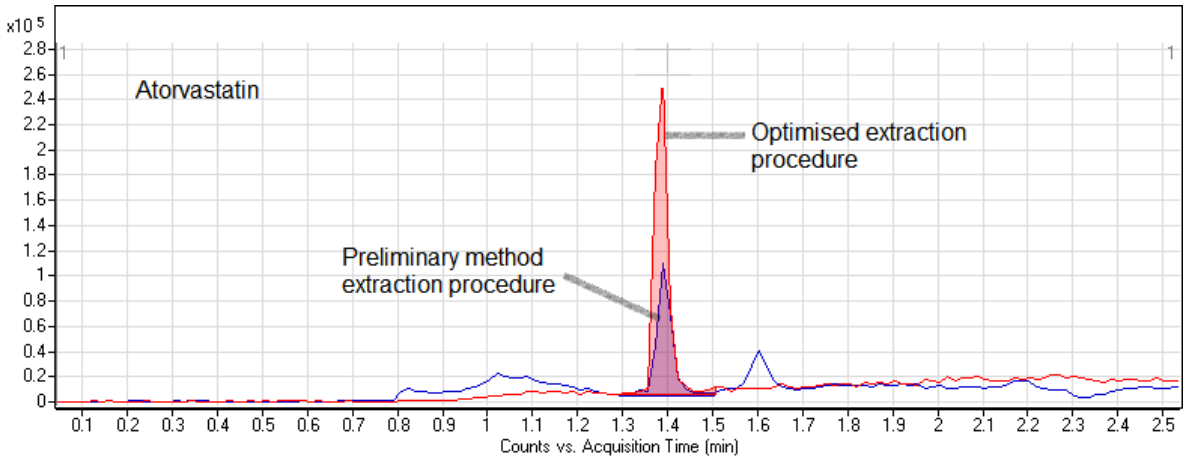


Figure 4.45 Representative LC-HRMS overlaid EIC's of atorvastatin DBS standard using the preliminary and optimised extraction procedures.

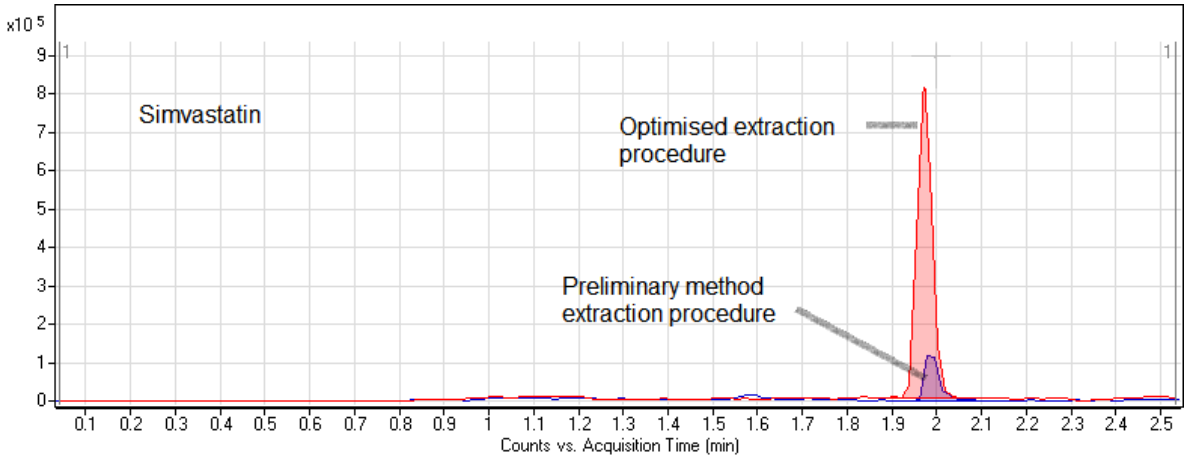


Figure 4.46 Representative LC-HRMS overlaid EIC's of simvastatin DBS standard using the preliminary and optimised extraction procedures.

4.5 Final DBS extraction protocols.

4.5.1 Extraction protocol for atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan

An 8 mm disc (~20 µl of blood) was punched from the centre of each DBS sample and transferred to a 1.5 ml micro-centrifuge tube. A 300 µl volume of methanol containing IS (20 ng/ml), atenolol D_7, was used for the extraction of atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan because of its optimum extraction efficiency and less interference. Tubes were vortexed for 1 min, sonicated for 30 mins in a temperature controlled ultrasonic bath at 40°C and centrifuged at 13200rpm for 10mins. 270 µl of each supernatant was transferred into a new microcentrifuge tube and dried under a gentle stream of N_2 gas. Dried residue was reconstituted with 150 µl of methanol/water (40:60, v/v) containing 0.1% formic acid. The final extracts were transferred into auto-sampler vials for LC-HRMS analyses. Figure 4.47 shows an overlaid EIC from a spiked DBS standard containing the 10 analytes and internal standard extracted using the optimised extraction procedure. Table 4.9 shows a comparison of the preliminary and optimised extraction procedures for the selected target analytes and internal standard.

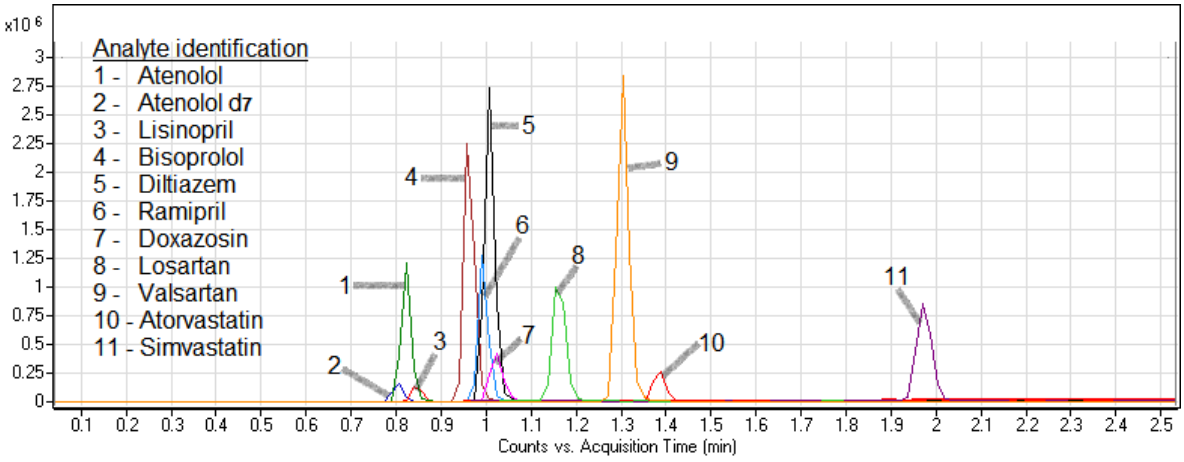


Figure 4.47 Representative LC-HRMS overlaid EIC's of a spiked DBS standard containing 20ng/ml of IS extracted with the optimised extraction procedure.

4.5.2 Final DBS extraction protocol for amlodipine

An 8-mm disc was punched from the centre of a 30 µl DBS sample and transferred into a clean 1.5 ml microcentrifuge tube. 300 µl of acetonitrile containing 20 ng/ml atenolol d_7 was added. 10µl of 0.5M sodium hydroxide was added to the extraction solvent. Tube was vortexed for 1 min, sonicated for 30 mins in a temperature controlled ultrasonic bath at 40°C and centrifuged at 13200 rpm for 10mins. 270 µl of supernatant was transferred into new microcentrifuge tubes and dried under a gentle stream of N_2 gas. Dried residue was reconstituted with 150 µl of acetonitrile/water (40:60, v/v) containing 0.1% formic acid and transferred into an autosampler vial for analysis. Figure 4.48 shows the process of analyte extraction from a spiked DBS standard.

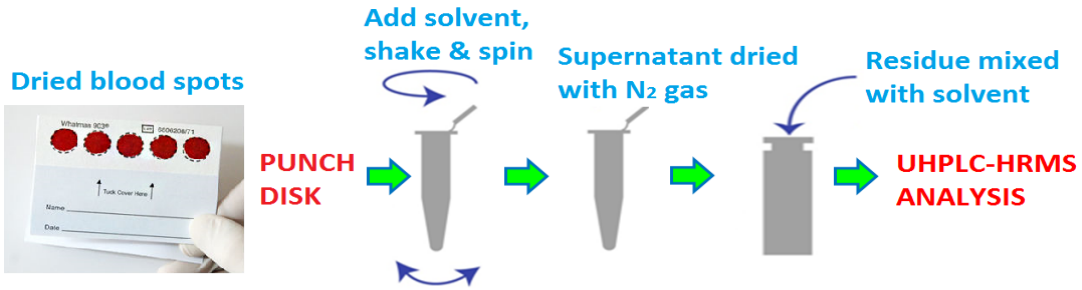


Figure 4.48 Schematic for the process of extraction of analytes from DBS cards using the optimised extraction procedure.

For volumetric absorptive microsampling (VAMS) samples, the whole substrate was used for extraction. Figure 4.49 shows the process of analyte extraction from a spiked VAMS standard.

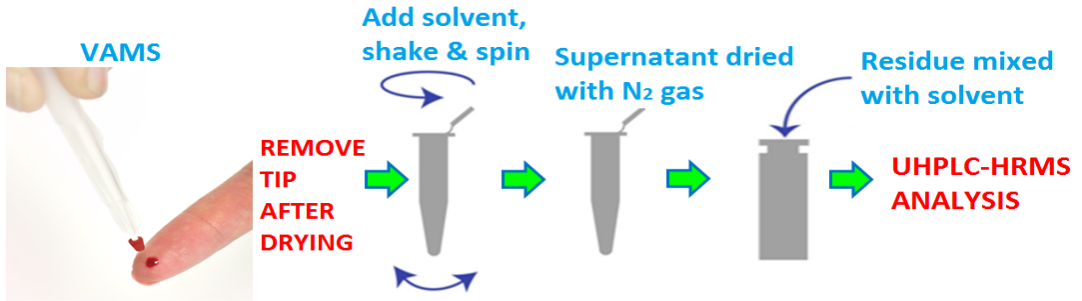


Figure 4.49 Schematic for the process of extraction of analytes from VAMS using the optimised extraction procedure.

Chapter 5 Validation of optimised LC-HRMS method using 903 sampling paper and volumetric absorptive microsampling (VAMS) device

There are international guidelines that bioanalytical methods used for measurement of analyte concentrations in human samples are well characterised, fully validated and documented to a specified standard to ensure reliable results (FDA, Guidance for industry, bioanalytical method validation 2001; Araujo, 2009; EU, Guideline on bioanalytical method validation, 2011; Lynch, 2016). This chapter therefore discusses the validation of the developed microsampling based LC-HRMS assay for the quantification of amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan on 903 sampling paper and volumetric absorptive microsampling (VAMS) device. A discussion of the various validation parameters investigated and a comparison of validation results for the two methods of microsampling is presented.

5.1 Introduction

Measurement of concentrations of analytes and their metabolites in biological samples (such as blood, plasma and serum) is a significant part of therapeutic drug monitoring (TDM). Derived data may be used to define or adjust the dose of the medication for the purposes of personalising the treatment for the patient by observing several factors that affect the efficacy and adverse effects of the drug. For example, the results of bioequivalence studies and TDM are required to make essential decisions supporting the safety and efficacy of a drug. Unreliable results could lead to the prescribing of wrong dose of the treatment to the patient with catastrophic consequence (Selinger et al., 2014). Thus, it is paramount that developed bioanalytical assays are fit for their intended purposes.

There are currently no official guidelines present for the validation of dried blood spot (DBS) assays (Enderle et al., 2016). Hence, the validation of the developed dried microsampling based LC-HRMS assay was conducted based upon FDA and EU

guidelines (FDA, Guidance for Industry: bioanalytical method validation 2001; EU, Guideline on bioanalytical method validation, 2011). Further considerations on the matrix and sampling specific validation parameters were based on the European Bioanalysis Forum (EBF) recommendation on the validation of bioanalytical methods for dried blood spots (Timmerman et al., 2011). The main characteristics of the developed assay that are crucial to ensure that performance are acceptable and analytical results are reliable were investigated. As per the FDA and EU guidelines, the essential parameters that need to be defined to ensure the acceptability of a bioanalytical method are precision, accuracy, sensitivity, selectivity, linearity, recovery, stability and matrix effects. Parameters based on EBF recommendations investigated include blood spot volume and the hematocrit (Hct) effect known to affect (drying time, homogeneity, diffusion and assay reproducibility) in DBS quantitative assays.

Different methods of blood microsampling have been developed recently to overcome the drawbacks of conventional DBS sampling (Spooner et al., 2015; Ye and Gao, 2017). Examples of these drawbacks include the difficulties with self-use, precise volume sampling and the effect of variability in individual hematocrit levels and their impact on the spot size and formation. One of such novel devices is volumetric absorptive microsampling (VAMS), which is discussed in Chapter 3, section 3.3.2. VAMS is designed to sample a fixed 10µl or 20µl volume of blood by sorption regardless of the blood hematocrit. The developed LC-HRMS assay was therefore validated using the conventional 903 sampling paper as a means of taking a microvolume of blood and the 10µl version of VAMS using spiked fresh human blood. Following successful validation, they were used for volunteer blood sample collection and subsequent analyses of the collected samples.

5.2 Method validation

For the purposes of validation studies, three concentrations were chosen for the independent preparation of quality control samples (QCs) at the low, medium and high concentration levels for each target drug and run alongside calibration standards as detailed in Table 4.7. To demonstrate that the developed bioanalytical method was fit

for purpose, validation was conducted based upon FDA and EU guidelines (FDA, 2001; EU, 2011). The selectivity, linearity, sensitivity, intra and inter-assay accuracy and precision, limit of quantification (LOQ), matrix effects, hematocrit effects and stability were determined for amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan on the 903 sampling paper and the VAMS device. Since amlodipine required a different extraction procedure from spiked blood samples, validation on the two methods of microsampling were performed separately for amlodipine.

5.2.1 Selectivity

Selectivity was evaluated to demonstrate that the developed LC-HRMS assay could differentiate the target analytes and internal standard (IS) from endogenous components in the 903 sampling card and VAMS device as well as the blood matrix. Selectivity was determined using three (3) individual sources of blank human whole blood. Possible interference from the matrix was investigated by the analyses of blank blood samples and target analyte spiked blood samples collected on 903 sampling paper and VAMS device.

Using the accurate masses for the 11 cardiovascular drugs and internal standard, determined by means of the qualitative analysis software version 4.00 (Mass calculator, Agilent Technologies) in Chapter 4, section 4.2.2, selectivity was evaluated by comparing extracted ion chromatograms (EICs) derived at the limit of quantification (LOQ) from a 903 sampling paper and VAMS calibration standard for each target analyte and the internal standard with those obtained from blank blood samples prepared on 903 sampling paper and VAMS device. A narrow mass extraction window of 5ppm was used to demonstrate enhanced selectivity. A representative proof of enhanced selectivity for atenolol on VAMS using mass tolerance filters of (a) ±200 ppm, (b) ±100 ppm and (c) ±5 ppm is shown in Figure 5.1.

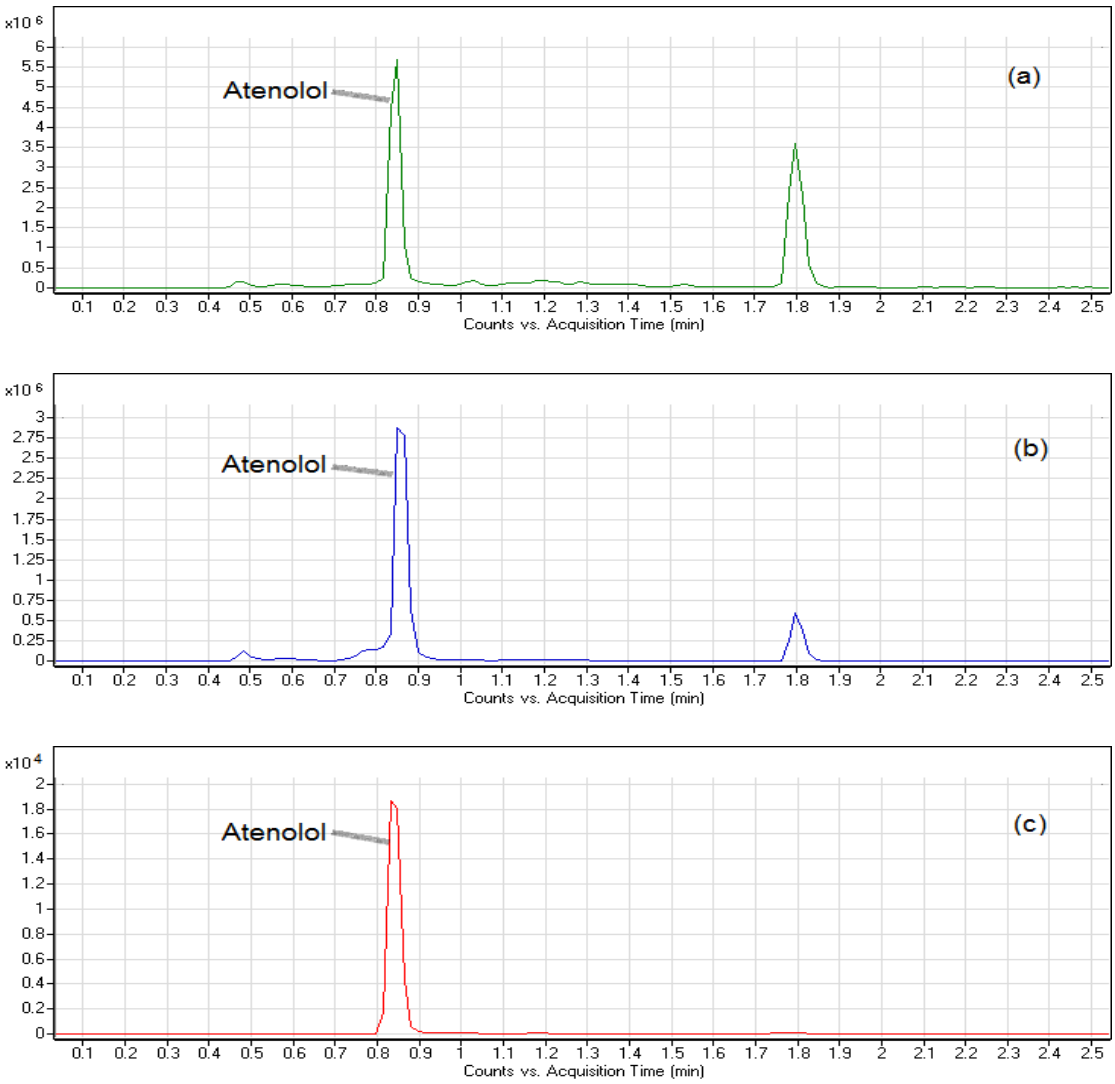
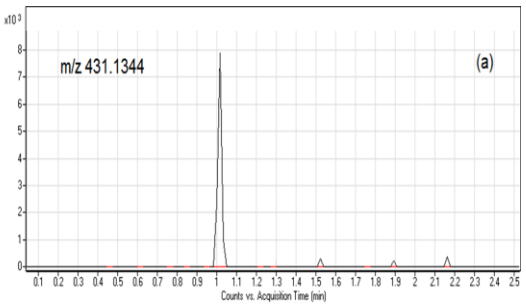


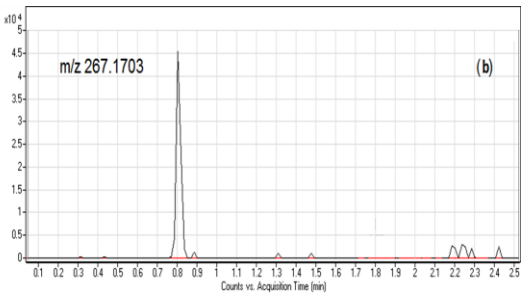
Figure 5.1 Demonstration of enhanced selectivity at the LOQ of atenolol with m/z 267.1703 using mass tolerance filters of (a)±200 ppm, (b)±100 ppm and (c)±5 ppm.

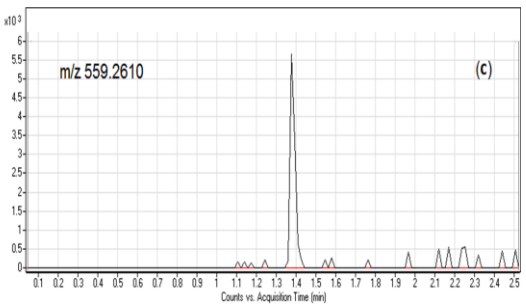
A side by side comparison of representative EICs at the LOQ for each analyte and internal standard on 903 sampling paper and VAMS is shown in Figures 5.2(a) – (l). One internal standard was used in the developed LC-HRMS assay because the introduction of several compounds as IS could lead to ionisation competition with the analytes of interest at the ESI source of the mass spectrometer resulting in additional matrix effects. The protonated molecule $[M+H]^+$ gave a high response for atenolol at m/z 267.1703, atorvastatin at m/z 559.2610, bisoprolol at m/z 326.2326, diltiazem at m/z 415.1686, doxazosin at m/z 452.1928, lisinopril at m/z 406.2336, losartan at m/z 423.1695, ramipril at m/z 417.2384, valsartan at m/z 436.2343 and atenolol d_7

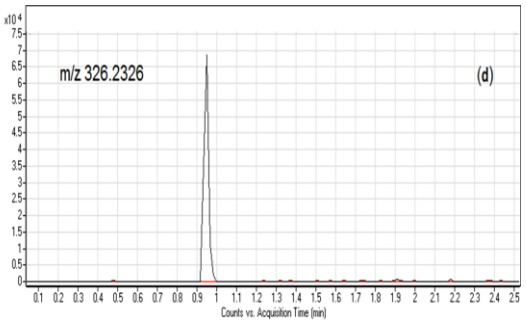
(internal standard) at m/z 274.2143. The sodium adduct ion $[M+Na]^+$ showed the highest signal intensity for amlodipine at m/z 431.1344 and simvastatin at m/z 441.2611. The sodium adduct ion for amlodipine and simvastatin were stable and gave reproducible response and thus was used for quantification. The LC-HRMS method showed good selectivity because the EICs revealed that no interfering peaks were observed at the retention times for each of the eleven drugs and IS investigated using the two methods of microsampling.

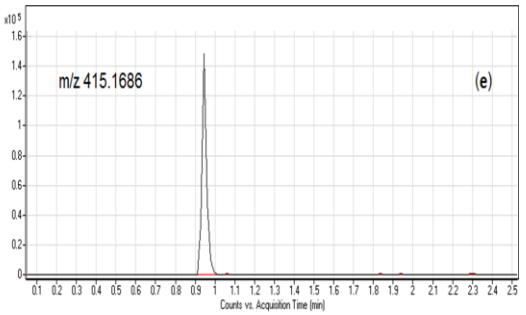
903 sampling paper



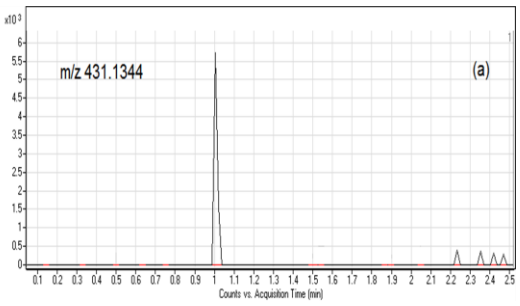


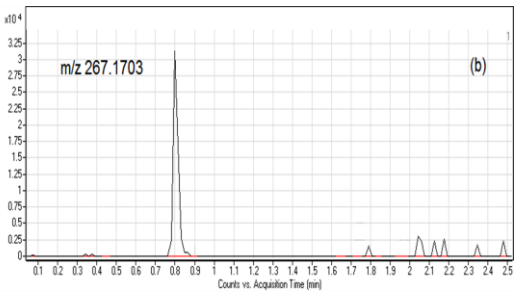


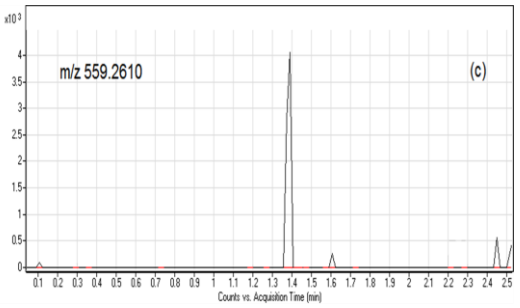


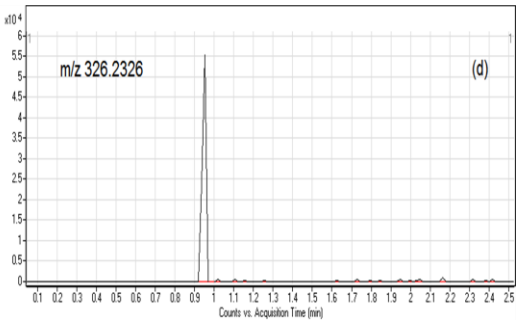


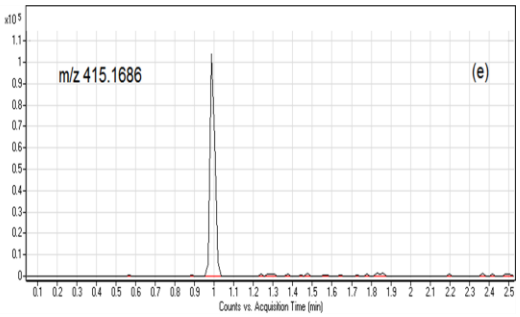
VAMS



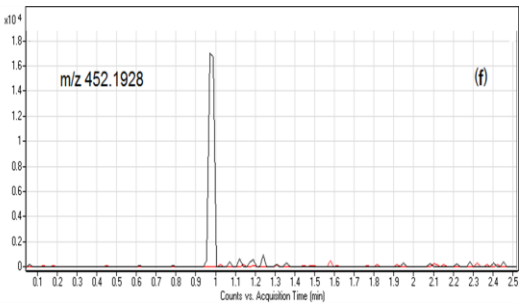


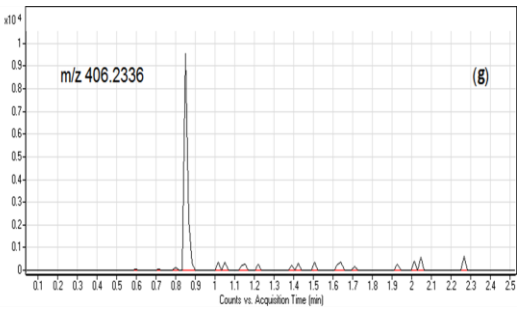


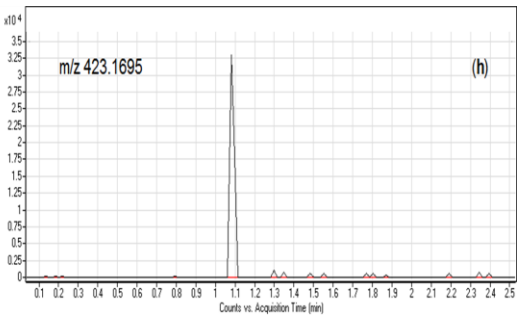


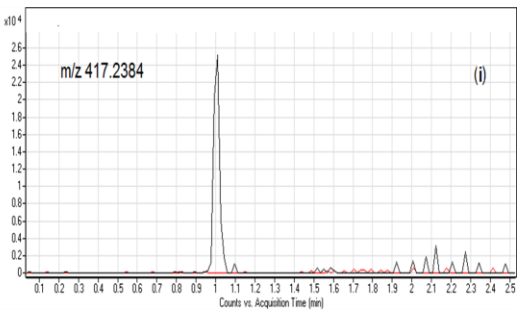


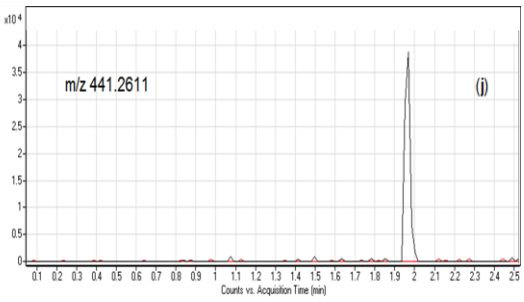
903 sampling paper continued



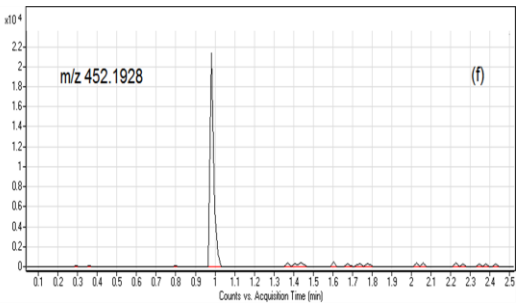


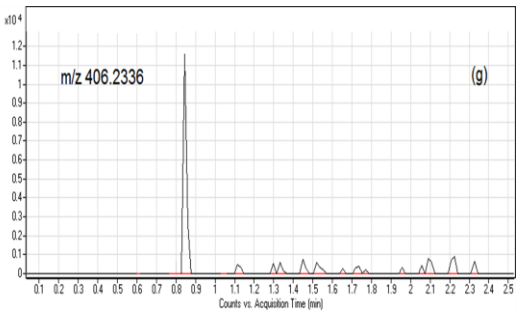


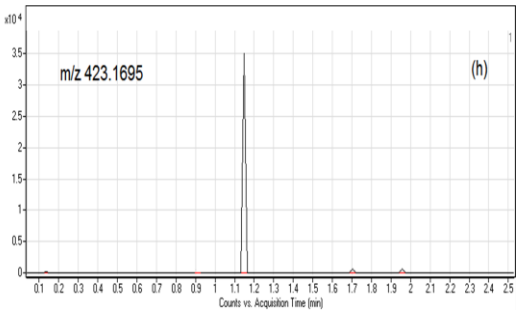


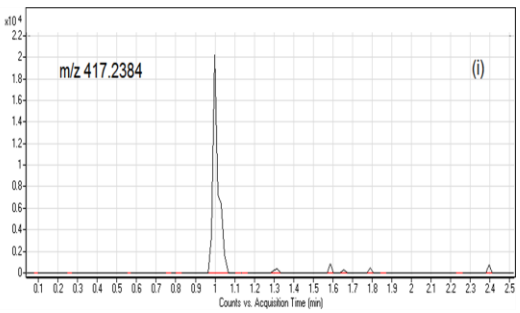


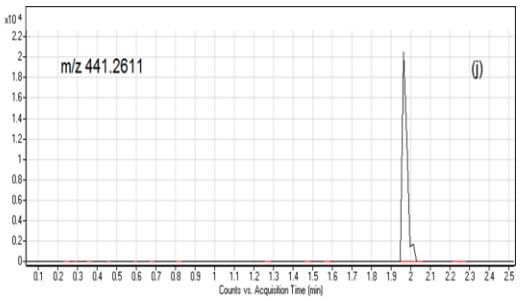
VAMS continued











903 sampling paper continued VAMS continued

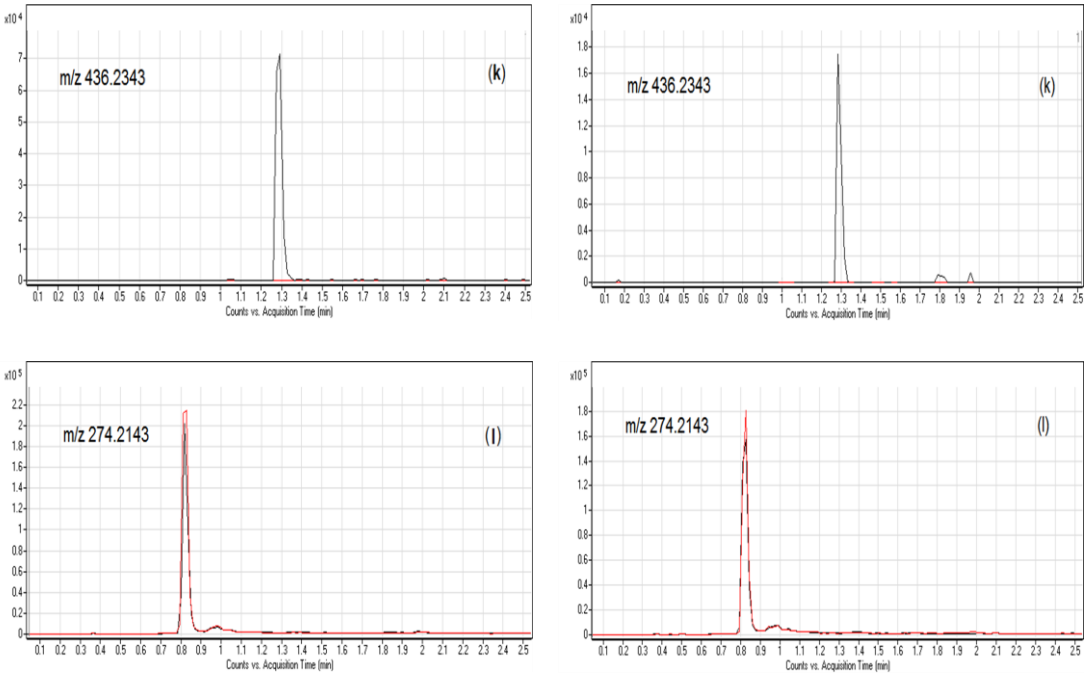


Figure 5.2 (a) – (l). A side by side representative LC-HRMS extracted (5ppm) ion chromatogram (EIC) of an extracted blank blood sample (red) and a calibration standard at the LOQ spiked with the eleven target drugs (black) on 903 sampling paper and VAMS. (a) amlodipine (b) atenolol (c) atorvastatin (d) bisoprolol (e) diltiazem (f) doxazosin (g) lisinopril (h) losartan (i) ramipril (j) simvastatin (k) valsartan and (l) atenolol d7 (internal standard).

5.2.2 Linearity and sensitivity

Replicate (n = 6) analyses of calibration standards prepared on 903 sampling paper and VAMS were separately run per day over the three days. A calibration plot for each target analyte/IS peak area ratio against nominal analyte concentration was produced and an equally-weighted linear regression was applied. The limit of quantification of amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan in the 903 sampling paper and VAMS extracts was defined by the lowest concentration that gave a signal-to-noise (S/N) ratio greater than or equal to 10. Back calculations gave relative errors less than 15% (typically between 2 and 10% for VAMS and between 2 and 14% for 903 sampling paper over the appropriate calibration range for each drug. The data (slope, intercept and the mean correlation coefficient R^2 and LOQ) for each drug is presented in Table 5.1a and 5.1b

for 903 sampling paper and VAMS respectively. The LOQ data shows comparable results for 903 sampling paper and VAMS. These results demonstrate that the sensitivity of VAMS is good taking into consideration the fact that amount of blood sample extracted was 10µl compared to ~20µl on 903 sampling paper. The results also demonstrate that the assay is sensitive enough for the determination of very low levels of the drugs in volunteer samples. The capability of the assay to quantify at such low concentrations will prevent the generation of false negative results. The high sensitivity reported for the target drugs also demonstrate the robustness of the extraction procedure. For example, in the quantification of bisoprolol, ramipril and simvastatin from DBS, Lawson et al (2013) reported sensitivities of 0.5ng/ml, 1ng/ml and 5ng/ml respectively, compared to 0.1ng/ml for all three drugs with this multi-drug LC-HRMS assay.

Table 5.1 (a) Linearity and sensitivity data for the eleven cardiovascular drugs in 903 sampling paper.

Drug	Range (ng/ml)	y = ax + b	R^2	LOQ (ng/ml)
Amlodipine	0.5 - 100	y = 0.004x + 0.043	0.993 ± 0.004	0.5
Atenolol	10 - 1500	y = 0.0044x - 0.047	0.997 ± 0.001	10
Atorvastatin	0.5 - 100	y = 0.0014x + 0.0244	0.986 ± 0.013	0.5
Bisoprolol	0.1 - 100	y = 0.019x + 0.034	0.994 ± 0.003	0.1
Diltiazem	0.5 - 600	y = 0.016x + 0.053	0.997 ± 0.002	0.5
Doxazosin	0.1 - 100	y = 0.016x + 0.033	0.992 ± 0.005	0.1
Lisinopril	0.1 - 100	y = 0.002x + 0.031	0.978 ± 0.007	0.1
Losartan	5 - 1000	y = 0.004x + 0.0713	0.995 ± 0.002	5
Ramipril	0.1 - 100	y = 0.025x + 0.018	0.997 ± 0.002	0.1
Simvastatin	0.1 - 100	y = 0.013x + 0.081	0.996 ± 0.003	0.1
Valsartan	50 - 4000	y = 0.002x - 0.139	0.994 ± 0.003	50

The following equation was used; $y = ax + b$ Equation 5.1

Where (y) is the ratio of analyte to I.S response, (a) is the slope, (x) represents the concentration and (b) is the y intercept.

Table 5.1 (b) Linearity and sensitivity data for the eleven cardiovascular drugs in VAMS.

Drug	Range (ng/ml)	y = ax + b	R^2	LOQ (ng/ml)
Amlodipine	0.5 - 100	y = 0.007x + 0.086	0.990 ± 0.002	0.5
Atenolol	10 - 1500	y = 0.0074x - 0.136	0.992 ± 0.001	10
Atorvastatin	0.5 - 100	y = 0.0033x + 0.023	0.997 ± 0.001	0.5
Bisoprolol	0.1 - 100	y = 0.0097x + 0.096	0.996 ± 0.002	0.1
Diltiazem	0.5 - 600	y = 0.008x + 0.224	0.995 ± 0.003	0.5
Doxazosin	0.1 - 100	y = 0.0067x + 0.067	0.992 ± 0.001	0.1
Lisinopril	0.1 - 100	y = 0.0013x + 0.021	0.985 ± 0.004	0.1
Losartan	5 - 1000	y = 0.0024x + 0.110	0.993 ± 0.007	5
Ramipril	0.1 - 100	y = 0.017x + 0.271	0.986 ± 0.001	0.1
Simvastatin	0.1 - 100	y = 0.016x + 0.215	0.988 ± 0.003	0.1
Valsartan	50 - 4000	y = 0.006x + 0.125	0.992 ± 0.001	50

5.2.3 Accuracy and precision

The accuracy and precision of the developed microsampling based LC-HRMS method were determined by intra and inter-day replicate analyses of six spiked 903 sampling paper and VAMS quality control (QC) samples containing the selected target analytes at the low, medium and high concentration levels on three separate days. Accuracy was expressed as the mean relative error (RE %) and precision was expressed as the coefficient of variation (CV %) and data obtained for both were within the predefined 15% limit for all concentrations in each run for all the target drugs with reference to FDA and EU guidelines. The overall variation in data between runs was also ≤15% for all target drugs on 903 sampling paper and VAMS and thus met international guideline criteria. A summary of the results is presented in Table 5.2a and Table 5.2b.

Table 5. 2 (a) Intra and inter-day accuracy and precision data for the eleven target cardiovascular drugs in 903 sampling paper extracts (n = 6 at all concentration levels).

Drug	Nominal conc. (ng/ml)	Measured conc. (ng/ml)	Accuracy (RE) %	Coefficient of variation (%)	
				Intra day	Inter day
Amlodipine	1	0.94	-5.53	8.91	6.94
	25	23.48	-6.07	13.55	7.83
	100	103.24	3.24	8.99	1.54
Atenolol	50	51.87	3.73	4.00	1.37
	500	498.02	-0.40	4.14	1.36
	1500	1517.51	1.17	2.22	1.24
Atorvastatin	1	1.05	5.43	4.06	5.93
	25	25.23	0.91	7.54	2.45
	100	100.69	0.69	7.19	2.41
Bisoprolol	1	1.09	9.09	2.63	3.50
	25	25.54	2.15	6.10	4.14
	100	102.42	2.42	3.21	2.76
Diltiazem	5	5.29	5.80	5.95	0.83
	100	98.64	-1.36	6.41	1.06
	600	611.85	1.97	2.03	1.49
Doxazosin	1	1.07	7.38	9.23	1.03
	25	25.59	2.36	3.74	3.58
	100	99.24	-0.76	3.89	2.78
Lisinopril	1	1.04	4.26	9.14	1.37
	25	24.91	-0.37	6.55	1.89
	100	100.31	0.31	6.61	2.19
Losartan	25	25.25	1.02	3.08	0.54
	250	248.57	-0.57	5.03	0.59
	1000	1014.66	1.47	5.99	1.62
Ramipril	1	1.01	1.23	4.29	2.60
	25	25.23	0.92	6.17	2.92
	100	101.76	1.76	4.60	3.28
Simvastatin	1	1.06	5.71	10.01	6.81
	25	25.13	0.51	6.43	0.86
	100	99.85	-0.15	3.98	2.11
Valsartan	250	242.75	-2.90	3.71	1.44
	2000	2078.29	3.91	3.32	3.44
	4000	4060.6	1.52	1.17	0.44

Table 5.2 (b) Intra and inter-day accuracy and precision data for the eleven target cardiovascular drugs in VAMS extracts (n = 6 at all concentration levels).

Drug	Nominal conc. (ng/ml)	Measured conc. (ng/ml)	Accuracy (RE)%	Coefficient of variation (%)	
				Intra day	Inter day
Amlodipine	1	1.08	7.33	8.06	13.45
	25	27.47	3.02	9.90	3.68
	100	101.14	2.51	1.14	12.09
Atenolol	50	51.58	3.16	1.71	2.07
	500	499.02	-0.20	6.04	0.97
	1500	1518.72	1.25	2.99	1.71
Atorvastatin	1	1.05	5.41	7.16	3.56
	25	25.18	0.70	4.31	1.99
	100	101.27	1.27	5.96	0.27
Bisoprolol	1	1.06	5.94	5.98	4.73
	25	25.20	0.81	7.93	1.73
	100	100.43	0.43	4.71	1.16
Diltiazem	5	5.16	3.15	4.22	2.54
	100	100.26	0.26	6.87	1.40
	600	603.52	0.59	2.59	0.42
Doxazosin	1	1.04	3.51	4.10	1.30
	25	25.77	2.03	7.68	0.93
	100	100.79	2.51	7.06	1.81
Lisinopril	1	1.06	4.49	5.66	2.53
	25	25.16	0.63	9.18	1.75
	100	100.32	0.32	7.82	0.75
Losartan	25	25.75	2.98	3.84	1.76
	250	250.13	0.05	4.13	2.16
	1000	1013.88	1.39	9.88	0.48
Ramipril	1	1.04	4,44	8.18	3.03
	25	25.37	1.47	6.38	4.46
	100	99.35	-0.65	3.72	0.67
Simvastatin	1	1.05	5.18	7.28	0.97
	25	25.15	0.59	6.25	1.30
	100	99.84	-0.16	5.16	0.22
Valsartan	250	250.34	0.14	5.27	1.19
	2000	1971.85	-1.41	3.05	1.55
	4000	4086.14	2.15	7.76	0.29

5.2.4 Matrix effects

The effect of matrix due to constituents from the blood, as well as from within the 903 sampling paper and VAMS material may cause ionisation competition between analytes of interest and co-eluents (Tang and Kebarle, 1993). This was evaluated to ensure that the sensitivity and precision of the developed LC-HRMS assay was not compromised on either 903 sampling paper or VAMS. To assess the effect of matrix, blood samples were collected from three different sources. Replicate (n = 6) samples of the selected target analytes spiked in blank blood spot extracts from 903 sampling paper and VAMS to represent the low, medium and high concentrations were prepared. The prepared samples were compared with standards of equal concentration spiked into methanol/water (40:60, v/v) containing 0.1% formic acid for atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan. For amlodipine, the prepared samples were compared with standards of equal concentration spiked into acetonitrile/water (40:60, v/v) containing 0.1% formic acid. The matrix effect was calculated using equation 5.2.

$$\textit{Matrix Effects} = \left(\frac{B}{A} - 1\right) X\ 100 \qquad \text{Equation 5.2}$$

Where A represents the ratio of the target analyte/IS response from analyte spiked into pure solvent and B represents the ratio of target analyte/IS response from analyte spiked into extracted blank whole blood. The matrix effect data obtained for each target analyte investigated at the low, medium and high concentration levels of the calibration curve is presented in Table 5.3a and 5.3b for 903 sampling paper and VAMS respectively. No significant (<10%) matrix effects on the analyte signal due to endogenous components of blood or the sampling material was observed at the three tested concentrations of each target drug. These results demonstrate the robustness of the extraction procedure and the ionisation mechanism for these target analytes. However, comparing the results for 903 sampling paper and VAMS, it is noteworthy that the mean matrix effect data for lisinopril and simvastatin were significantly low on VAMS compared to 903 sampling paper (Tables 5.3a and 5.3b). The observed difference may be attributed to the constituents within the 903 sampling paper

causing ionisation competition with lisinopril and simvastatin at the ESI source of the MS.

Table 5.3 (a) Matrix effect results obtained for the eleven target drugs studied at the low, medium and high concentration levels on 903 sampling paper. n = 6 for each concentration.

Drug	Nominal conc. (ng/ml)	Matrix effect % (mean)	Precision (CV%)
Amlodipine	1	-3.32	5.66
	25	-3.25	6.13
	100	-1.99	4.41
Atenolol	50	-1.94	5.59
	500	0.84	2.03
	1500	-1.86	1.72
Atorvastatin	1	2.41	1.65
	25	1.25	1.93
	100	1.95	1.29
Bisoprolol	1	-1.39	2.17
	25	0.41	2.73
	100	0.67	0.98
Diltiazem	5	1.43	2.75
	100	0.06	3.03
	600	1.49	1.33
Doxazosin	1	0.60	2.76
	25	0.73	1.69
	100	-0.85	2.01
Lisinopril	1	8.91	4.55
	25	5.99	1.60
	100	2.54	2.33
Losartan	25	0.94	1.72
	250	2.07	1.51
	1000	0.51	0.93
Ramipril	1	0.35	2.86
	25	0.54	2.94
	100	1.98	0.34
Simvastatin	1	7.01	6.23
	25	-3.62	5.43
	100	-4.56	5.68
Valsartan	250	-1.12	2.71
	2000	-1.70	2.97
	4000	-2.84	1.50

Table 5.3 (b) Matrix effect results obtained for the eleven target drugs studied at the low, medium and high concentration levels on VAMS. n = 6 for each concentration.

Drug	Nominal conc. (ng/ml)	Matrix effect % (mean)	Precision (CV%)
Amlodipine	1	-1.21	4.72
	25	3.49	8.54
	100	-2.96	3.99
Atenolol	50	0.88	1.34
	500	3.39	2.52
	1500	2.20	3.81
Atorvastatin	1	1.80	2.49
	25	1.90	6.40
	100	3.87	4.20
Bisoprolol	1	0.91	4.78
	25	2.49	2.77
	100	0.66	1.23
Diltiazem	5	3.59	4.21
	100	1.19	1.87
	600	1.62	4.21
Doxazosin	1	-3.52	3.53
	25	-2.21	3.53
	100	-0.44	2.61
Lisinopril	1	0.99	2.74
	25	1.46	3.52
	100	3.66	4.76
Losartan	25	2.00	3.94
	250	-1.36	6.68
	1000	-2.46	7.45
Ramipril	1	-2.41	2.35
	25	-3.45	5.02
	100	1.91	5.01
Simvastatin	1	2.70	3.56
	25	0.66	2.86
	100	0.51	2.80
Valsartan	250	-1.17	5.87
	2000	1.01	0.02
	4000	0.99	0.08

5.2.5 Recovery of the 11 target analytes from 903 sampling paper and VAMS

Recovery (extraction efficiency) was determined using replicate (n = 6) samples prepared at the (low, medium and high) concentrations for the eleven target drugs from spiked 903 sampling paper and VAMS extracts. Recovery was assessed by comparing the ratios of analyte to IS response from 903 sampling paper and VAMS extracts with those obtained from blank blood spot extracts spiked with solution standards of equal concentration. Recovery was calculated using equation 5.3:

Percentage Recovery = $\left(\frac{B}{A}\right) X\ 100$ Equation 5.3, where B is analyte to IS response of 903 card or VAMS extract and A is analyte to IS response of post extraction blank 903 card or VAMS spiked extract. Recoveries for atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan and ramipril on 903 sampling paper and VAMS were consistent, with values between 77 and 103%. The high recoveries observed indicate analyte stability under the extraction conditions applied and good extraction. The overall mean recovery for amlodipine was 68% and 62% on 903 sampling paper and VAMS respectively and simvastatin was 67% on both. In contrast with previously published results, Lawson et al (2013) reported mean recoveries of 43% for simvastatin in dried blood spot extracts. Thus, simvastatin shows a significant increase in recovery (~35%) with the developed LC-HRMS assay. The observed difference is attributed to the robust extraction procedure developed for the extraction of the target drugs from DBS and VAMS samples. Compared to the extraction protocol documented by Lawson et al (2013) which used an extraction solvent volume of 150µl (Chapter 4, Table 4.9), the current extraction procedure uses - (a) a larger volume of extraction solvent (300µl) which ensures optimum extraction of simvastatin from DBS, (b) – it also include a dry down step which involves the concentration of extracted supernatant and subsequent reconstitution. This procedure produces cleaner extracts with less interference which reduces matrix effects and improve recovery. Valsartan is the only analyte that did not give comparable recoveries on the two sampling methods. A high extraction recovery of 95% was observed on 903 sampling paper, but on VAMS overall mean recovery was 47%. Possible reason for the difference may be poor extraction of

valsartan from the VAMS substrate. Recovery data for each target analyte at the low, medium and high concentration levels on 903 sampling paper and VAMS are summarised in Tables 5.4a and 5.4b.

Table 5.4 (a) Recovery data for the 11 target drugs extracted from 903 sampling paper at the low, medium and high concentration levels (n = 6).

Drug	Nominal conc. (ng/ml)	Recovery (%)	Standard Deviation (SD)	Precision (CV)
Amlodipine	1	65.94	8.21	12.44
	25	74.71	5.48	7.34
	500	65.35	3.85	5.89
Atenolol	50	89.13	6.53	7.32
	500	82.54	7.60	9.21
	1500	93.16	3.69	3.96
Atorvastatin	1	101.09	10.24	10.13
	25	95.43	7.25	7.60
	100	99.76	1.64	1.64
Bisoprolol	1	101.65	11.34	11.16
	25	99.19	5.68	5.73
	100	89.53	5.52	6.16
Diltiazem	5	98.08	12.42	12.67
	100	88.92	4.24	4.77
	600	85.05	1.80	2.11
Doxazosin	1	97.86	7.07	7.23
	25	97.37	5.00	5.14
	100	94.89	6.19	6.52
Lisinopril	1	87.43	9.08	9.32
	25	90.51	7.88	8.71
	100	85.39	4.65	6.17
Losartan	25	97.34	4.03	4.14
	250	94.27	10.25	10.88
	1000	87.1	4.61	5.30
Ramipril	1	97.08	7.15	7.37
	25	89.94	5.38	5.98
	100	92.96	3.36	3.62
Simvastatin	1	67.88	4.26	6.28
	25	64.74	5.97	9.22
	100	70.81	3.96	5.59
Valsartan	250	100.66	3.44	3.41
	2000	97.35	2.29	2.35
	4000	88.67	9.11	10.28

Table 5.4 (b) Recovery data for the 11 target drugs extracted from VAMS at the low, medium and high concentration levels (n = 6).

Drug	Nominal conc. (ng/ml)	Recovery (%)	Standard Deviation (SD)	Precision (CV)
Amlodipine	1	57.33	3.83	10.17
	25	62.18	1.88	6.87
	100	66.42	4.25	8.99
Atenolol	50	103.31	6.36	5.61
	500	106.17	2.65	2.34
	1500	98.95	1.26	1.28
Atorvastatin	1	101.01	12.79	9.27
	25	95.61	10.91	8.69
	100	91.64	4.85	5.29
Bisoprolol	1	88.41	6.82	6.29
	25	86.58	4.92	7.17
	100	88.02	0.81	1.39
Diltiazem	5	78.69	4.75	3.43
	100	82.50	7.30	13.90
	600	74.50	0.29	0.38
Doxazosin	1	89.66	2.28	2.26
	25	92.37	4.03	4.36
	100	86.64	3.03	4.83
Lisinopril	1	88.71	11.97	13.40
	25	84.16	11.33	9.92
	100	91.66	9.43	9.46
Losartan	25	93.78	6.23	6.64
	250	101.06	6.21	6.14
	1000	96.63	2.18	2.85
Ramipril	1	88.25	4.40	4.98
	25	90.25	7.74	7.50
	100	83.52	3.53	4.23
Simvastatin	1	62.87	13.18	8.09
	25	71.95	6.06	8.42
	100	66.94	1.91	2.85
Valsartan	250	39.89	5.80	3.82
	2000	53.02	3.68	1.47
	4000	48.44	2.55	2.43

5.2.6 Blood spot size investigation on 903 sampling paper

This investigation was conducted to demonstrate that after selection of a disc size for analyses, the quantitative results obtained were not affected by the volume of blood deposited or the size of the blood spot presuming there is uniformity in the spread of the spot on filter paper. This experiment was not applicable to the VAMS device since it samples a fixed 10µl volume of blood. To investigate the blood volume effect on the quantification of the eleven target analytes, replicate analyses (n = 6) were performed at medium and high concentrations for the target drugs using prepared 20, 30 and 40 µl blood spots. These spots had different diameters of approximately 7.2mm, 9.5mm and 12.0mm respectively, directly proportional to sample volume deposited. 8mm discs (approximately 20 µl of blood) were punched from the centre of the already prepared 20, 30 and 40 µl volume DBS standards. Extraction of the target drugs was performed using the procedure described in Chapter 4, section 4.5 prior to LC-HRMS analyses. Using a linear regression equation obtained from a calibration generated with 30 µl volume DBS, the analyte concentration of the extracts was determined.

Method precision and accuracy were assessed using extraction data from an 8 mm discs, sampled from the centre of the 20, 30 and 40 µl volume DBS prepared at the medium and high concentration levels for the eleven target analytes. Table 5.5 shows the intra-day precision and accuracy of the method evaluated using 6 determinations for each concentration level. Results obtained for accuracy and precision were less than 15% and therefore met international guideline criteria. These experiments were performed to demonstrate that results obtained were not dependent on the size of the blood spot collected. Analysing a fixed sample size disc should produce extract data which is directly proportional to the concentration of the target analyte in the original blood sample. The results in Table 5.5 affirm that within experimental error for each concentration range the data from 8 mm discs is the same regardless of sample volume chosen.

Table 5.5 Impact of dried blood spot size on accuracy and precision of assay at the medium and high concentrations for each target drug (n = 6).

Amlodpine concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
25	40	24.87 ± 1.38	0.53	5.56
	30	25.59 ± 1.61	2.35	6.29
	20	25.99 ± 0.72	3.97	2.77
100	40	103.59 ± 5.76	3.59	5.56
	30	100.50 ± 4.59	0.50	4.57
	20	103.04 ± 3.00	3.04	9.71
Atenolol concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
500	40	523.84 ± 9.03	4.77	1.72
	30	489.10 ± 19.27	2.18	3.94
	20	494.26 ± 17.82	1.15	3.61
1500	40	1492.36 ± 29.02	0.51	8.65
	30	1456.05 ± 12.75	2.93	0.88
	20	1590.79 ± 16.73	6.05	1.05
Atorvastatin concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
25	40	24.33 ± 2.25	2.26	9.24
	30	24.55 ± 2.06	1.81	8.39
	20	24.80 ± 3.11	0.79	12.54
100	40	100.94 ± 3.90	0.94	3.86
	30	98.32 ± 2.83	1.68	2.88
	20	100.35 ± 2.75	0.35	2.74
Bisoprolol concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
25	40	25.41 ± 2.62	1.65	10.33
	30	22.96 ± 0.71	8.17	3.07
	20	25.25 ± 1.07	0.99	4.22
100	40	99.93 ± 1.41	0.07	1.42
	30	101.52 ± 7.10	1.52	6.99
	20	105.27 ± 2.95	5.27	2.8
Diltiazem concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
100	40	92.51 ± 5.40	7.49	5.84
	30	93.18 ± 6.23	6.82	6.69
	20	91.70 ± 5.59	8.3	6.1
600	40	595.19 ± 14.09	0.8	5.73
	30	590.04 ± 10.84	1.66	1.84
	20	615.61 ± 4.35	2.6	0.71

Table 5.5 continued

Doxazosin concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
25	40	25.37 ± 1.19	1.46	4.68
	30	26.26 ± 0.96	5.03	3.64
	20	25.71 ± 1.04	2.83	4.05
100	40	100.77 ± 5.74	0.77	5.69
	30	98.96 ± 2.17	1.04	2.2
	20	103.19 ± 1.69	3.19	1.63

Lisinopril concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
25	40	24.01 ± 1.02	3.96	4.27
	30	26.47 ± 2.39	5.87	9.04
	20	25.81 ± 2.18	3.25	8.44
100	40	102.00 ± 7.91	2.00	7.75
	30	100.21 ± 5.04	0.21	5.03
	20	107.93 ± 3.41	7.93	3.16

Losartan concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
250	40	251.40 ± 3.90	0.56	1.55
	30	251.87 ± 2.51	0.75	1.00
	20	250.16 ± 6.41	0.07	2.56
1000	40	1012.38 ± 43.75	1.24	4.32
	30	987.23 ± 20.32	1.28	2.06
	20	1017.71 ± 14.84	1.77	1.46

Ramipril concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
25	40	24.80 ± 1.06	0.81	4.26
	30	25.84 ± 0.95	3.36	3.69
	20	24.67 ± 0.82	1.33	3.31
100	40	101.18 ± 4.86	1.18	4.81
	30	99.59 ± 1.09	0.41	1.10
	20	102.95 ± 2.18	2.95	2.12

Simvastatin concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
25	40	25.46 ± 1.77	1.82	6.95
	30	25.57 ± 0.88	2.27	3.44
	20	25.14 ± 0.54	0.58	2.16
100	40	105.55 ± 6.18	5.55	5.86
	30	100.84 ± 3.11	0.84	3.08
	20	100.91 ± 1.87	0.91	1.86

Table 5.5 continued

Valsartan concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
2000	40	1942.50 ± 17.02	2.87	0.88
	30	1943.26 ± 11.80	2.84	0.61
	20	1988.18 ± 33.18	0.59	4.18
4000	40	4038.38 ± 77.57	0.96	1.92
	30	4075.53 ± 83.71	1.89	2.05
	20	4149.79 ± 26.93	3.74	0.65

5.2.7 Evaluation of the effects of different hematocrit levels on data from 903 sampling paper and VAMS

The hematocrit (Hct) level represents the relative volume of red blood cells (RBC) in blood. It has a direct effect on the viscosity of blood, which in turn affects the spread of blood on cellulose based paper and the level of drug absorbed into the red blood cells (Denniff and Spooner, 2010, De Vries et al., 2013). Blood with high Hct (due to the high cellular composition) is more viscous and leads to the formation of small spots on DBS cards. The Hct range varies according to age for healthy adult males and females. It is 40 – 54% and 36 – 48% respectively (Walker et al., 1990). Hct values may however deviate from these ranges in certain disease states e.g. anaemia and polycythaemia. An Hct value of 45% was chosen to represent the average value expected in the target population planned for this study. The bias caused by the hematocrit variability of the DBS sample has been considered a critical parameter affecting quantitative DBS analyses (O'mara et al 2011; Fan et al 2012). This has led to the development of novel devices like VAMS, which is reported to be independent of the hematocrit effect (Spooner et al 2015; De Kesel et al 2015; Mano et al 2015). Hence the influence of hematocrit on assay performance was evaluated at the low, medium and high concentrations of each target drug (n = 6) using 30µl spots on 903 sampling paper and 10µl volume for VAMS device with an adjusted Hct of 35, 45 and 55% to cover the range for the target population.

5.2.7.1 Comparison of the effects of different Hct levels on the results obtained from spiked blood samples extracted from 903 paper and VAMS

Blank human whole blood was centrifuged at 10,000g for 12 minutes (Koster et al., 2015; Hettich Laboratory Limited, determination of the hematocrit value by centrifugation, 2016). The plasma generated was transferred into a clean microcentrifuge tube. The RBC suspension and plasma were mixed in proportions (35:65, v/v), (45:55, v/v) and (55:45, v/v) to give whole blood with an adjusted Hct of 35, 45 and 55% respectively. These were used to prepare calibration samples on 903 sampling paper and VAMS device for the eleven target analytes at the blank, low, medium and high concentration ranges. 30µl of each prepared standard were spotted on 903 sampling paper and allowed to dry for 3 hours. 8mm disc were punched from the centre of each spot and extracted using the procedure described in Chapter 4, section 4.5. In the case of VAMS, 10µl of each prepared standard were sampled on the polymeric tip of the device, dried and extracted using the procedure as described in Chapter 4, section 4.5.

Concentrations of extracts were determined using a linear regression equation generated from calibration data produced from standards prepared with the 45% Hct on 903 sampling paper and VAMS. For the samples prepared on 903 sampling paper, a decrease in size of spots formed was observed with increasing Hct value across the range of 35% to 55% investigated. The results from the hematocrit investigation are shown in Tables 5.6a and 5.6b. Results from the VAMS device, gave accuracy (RE%) and precision (CV%) values within the pre-defined limit of ≤ 15% (De Vries et al., 2013) at all hematocrit levels for each tested analyte concentration. Results for 903 sampling paper shows only one marginal increase over the 15% limit at 15.3% for atorvastatin at the 35% Hct level. Possibly, because a punched sample on 903 sampling paper was used. For 903 sampling paper, data from the 35% and 55% Hct levels show significant differences, that is negative values at the 35% Hct level and positive values for the 55% Hct levels as expected. For VAMS the differences are much reduced as expected. This demonstrates that quantitative analytical data collected on the VAMS device will not be affected by interindividual variability in hematocrit values for the hematocrit range

investigated for the 11 target drugs. In contrast with VAMS, results from the 903 sampling paper indicates that at extreme hematocrit values, hematocrit effects may be significant on 903 sampling paper for the target drugs investigated, when a punched sample is used due to interindividual variation which may affect the quantitative results. However, taking into consideration the patient population used for this research, there is no evidence that heart disease affects a patient's blood hematocrit level. Thus, the overall results demonstrate the acceptability of the developed microsampling based LC-HRMS method for quantitative analyses of CVD drugs. The results also demonstrate the robustness of the extraction procedure, as different hematocrits do not result in differences in matrix effects.

Table 5.6 (a) Influence of Hematocrit on the accuracy (RE %) of analyte quantification for 903 sampling paper presented as the difference from the analyte/internal standard peak area ratio at the 45% Hct level. Precision (CV %) values for each tested concentration are shown in brackets (n = 6).

		Hematocrit		
Drug	Nominal conc. (ng/ml)	35%	45%	55%
Amlodipine	1	-3.8% (9.4%)	Normalised (6.2%)	4.1% (8.9%)
	25	3.6% (5.1%)	Normalised (8.8%)	2.2% (4.9%)
	100	-9.5% (4.3%)	Normalised (3.0%)	11.8% (10.6%)
Atenolol	50	-7.4% (4.1%)	Normalized (5.9%)	8.8% (3.5%)
	500	-7.6% (1.5%)	Normalized (2.6%)	14.5% (5.0%)
	1500	-8.4% (3.6%)	Normalized (1.9%)	6.4% (2.1%)
Atorvastatin	1	-4.1% (6.04)	Normalised (10.1%)	4.0% (12.8%)
	25	-15.3% (2.67)	Normalised (6.6%)	12.5% (7.7%)
	100	-14.6% (3.65)	Normalised (3.0%)	2.2% (2.6%)
Bisoprolol	1	-10.2% (9.2%)	Normalised (5.1%)	11.2% (10.5%)
	25	-12.4% (4.6%)	Normalised (15.1%)	13.8% (5.5%)
	100	-14.4% (7.3%)	Normalised (7.0%)	7.9% (4.7%)
Diltiazem	5	-9.4% (6.3%)	Normalised (10.1%)	13.1% (5.5%)
	100	-7.1% (10.6%)	Normalised (6.6%)	13.9% (2.8%)
	600	-12.3% (2.4%)	Normalised (3.0%)	10.5% (1.5%)
Doxazosin	1	-14.1% (5.2)	Normalised (10.3%)	3.1% (7.8%)
	25	-3.02% (4.6%)	Normalised (3.9%)	2.8% (2.1%)
	100	-7.9% (4.2)	Normalised (5.5%)	5.7% (3.3%)
Lisinopril	1	-10.7% (10.3%)	Normalised (10.1%)	8.5% (6.1%)
	25	-12.8% (4.7%)	Normalised (6.6%)	3.4% (8.7%)
	100	-6.6% (10.5%)	Normalised (3.0%)	10.3% (10.1%)
Losartan	25	-14.3% (7.02%)	Normalised (5.0%)	7.14% (6.6%)
	250	-9.8% (2.2%)	Normalised (7.9%)	10.9% (6.0%)
	1000	-9.34% (5.6%)	Normalised (6.1%)	2.7% (1.9%)
Ramipril	1	-10.6% (14.2%)	Normalised (6.1%)	12.8% (7.8%)
	25	-10.1% (4.1%)	Normalised (5.9%)	7.2% (6.2%)
	100	-9.1% (1.7%)	Normalised (6.2%)	1.4% (1.37%)
Simvastatin	1	1.5% (12.3%)	Normalised (10.1%)	8.4% (3.8%)
	25	-13.3% (6.0)	Normalised (6.6%)	11.5% (7.4%)
	100	-3.1% (2.9%)	Normalised (3.0%)	9.5% (8.9%)
Valsartan	250	-11.5% (5.5%)	Normalised (1.6%)	5.4% (8.21%)
	2000	-7.6% (7.2%)	Normalised (8.2%)	13.6% (11.53%)
	4000	-11.4% (6.01%)	Normalised (12.5%)	11.6% (3.66%)

Table 5.6 (b) Influence of Hematocrit on the accuracy (RE %) of analyte quantification for VAMS presented as the difference from the analyte/internal standard peak area ratio at the 45% Hct level. Precision (CV %) values for each tested concentration are shown in brackets (n = 6).

		Hematocrit		
Drug	Nominal conc. (ng/ml)	35%	45%	55%
Amlodipine	1	-3.47% (9.77%)	Normalised (7.27%)	3.21% (9.59%)
	25	-5.29% (5.65%)	Normalised (12.24%)	-2.48% (4.53%)
	100	2.41% (7.36%)	Normalised (4.83%)	2.89% (5.42%)
Atenolol	50	-6.52% (4.03)	Normalized (7.15%)	-5.80 (4.13)
	500	5.19% (4.27)	Normalized (4.38%)	5.10 (3.15)
	1500	-1.13 (8.45)	Normalized (4.72%)	-2.19 (4.74)
Atorvastatin	1	-0.72% (12.22%)	Normalised (12.04%)	5.82% (9.17%)
	25	0.08% (5.99%)	Normalised (6.35%)	0.74% (4.83%)
	100	-3.66% (7.58%)	Normalised (7.66%)	-4.83% (6.46%)
Bisoprolol	1	-5.26% (6.4%)	Normalised (12.44%)	2.01% (4.3%)
	25	0.32% (5.2%)	Normalised (3.12%)	-0.68% (2.2%)
	100	-1.14% (2.8%)	Normalised (3.28%)	-0.40% (2.7%)
Diltiazem	5	2.43% (0.17%)	Normalised (0.05%)	-0.06% (4.85%)
	100	-1.06% (0.01%)	Normalised (0.01%)	0.87% (0.62%)
	600	0.16% (0.03%)	Normalised (0.02%)	-0.16% (2.62%)
Doxazosin	1	-3.08% (6.3%)	Normalised (12.44%)	-3.81% (4.40%)
	25	2.43% (7.53%)	Normalised (3.12%)	-4.69% (4.84%)
	100	-0.23% (1.14%)	Normalised (3.28%)	-0.20% (3.15%)
Lisinopril	1	-0.93% (10.03%)	Normalised (13.44%)	2.93% (13.60%)
	25	4.35% (10.17%)	Normalised (3.37%)	3.65% (8.45%)
	100	-5.90% (9.32%)	Normalised (4.64%)	-0.95% (3.89%)
Losartan	25	-1.42% (0.09%)	Normalised (5.69%)	-0.43% (8.53%)
	250	4.20% (0.05%)	Normalised (6.11%)	2.91% (3.07%)
	1000	-1.85% (0.02%)	Normalised (0.97%)	-1.63% (1.72%)
Ramipril	1	1.84% (3.16%)	Normalised (2.15%)	-3.60% (4.82%)
	25	3.22% (8.05%)	Normalised (9.7%)	2.00% (6.2%)
	100	0.21% (1.83%)	Normalised (2.38%)	-0.55% (1.82%)
Simvastatin	1	1.13% (3.68%)	Normalised (8.16%)	-0.21% (9.89%)
	25	5.03% (6.08)	Normalised (3.7%)	-2.70% (6.6%)
	100	-2.42% (2.9%)	Normalised (3.2%)	-5.70% (4.6%)
Valsartan	250	-1.45% (2.28%)	Normalised (1.15%)	-1.05% (2.07%)
	2000	-0.52% (3.37%)	Normalised (3.48%)	-2.81% (3.08%)
	4000	-0.43% (3.97%)	Normalised (2.61%)	-0.98% (2.23%)

5.2.8 Stability of dried blood samples on 903 sampling paper and VAMS

The stability of dried blood samples after 10 weeks of storage at room temperature in 903 sampling paper and 8 weeks of storage in VAMS was assessed to demonstrate the possibility to collect 903 card and VAMS samples in batches. This was done by the replicate analyses (n = 6) of blood samples on 903 sampling paper and VAMS containing amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan at the low, medium and high concentrations. Using the extraction procedure described in Chapter 4, (section 4.5.1 and 4.5.2), 8mm diameter discs were punched from the 903 sampling paper calibration standards at the low, medium and high concentrations of the 11 target drugs and analysed. For VAMS the whole sample was used for extraction. No significant changes in concentrations were observed after 10 weeks of storage in 903 paper and 8 weeks of storage in VAMS at the low, medium and high concentration levels of target drugs as shown in Table 5.7a and 5.7b**.** These results demonstrate that for spiked samples the eleven target drugs are stable in 903 sampling paper for 10 weeks and in VAMS for 8 weeks when stored at room temperature. Studies in this laboratory have shown similar stability for atenolol, bisoprolol, simvastatin and valsartan in 'real' DBS samples from volunteers (Lawson et al., 2013). It also affirms the feasibility of using dried microsampling methodology in resource limited areas. This is because samples may have to be collected in remote areas of the country and will take several days or even weeks to be transported back to the laboratory for analyses.

Table 5.7 (a) Accuracy, precision and quantification of 903 sampling paper assay at the low, medium and high concentrations for each target drug after 10 weeks of storage at room temperature (n = 6).

Drug	Concentration in whole blood (ng/ml)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
Amlodipine	1	0.99 ± 0.05	6.43	7.59
	25	25.21 ± 1.12	4.43	6.28
	100	99.01 ± 4.36	-2.10	3.76
Atenolol	50	59.90 ± 2.58	12.06	1.11
	500	464.47 ± 23.96	-2.52	2.58
	1500	1572.70 ± 39.71	4.69	0.85
Atorvastatin	1	1.10 ± 0.08	1.34	11.69
	25	27.64 ± 1.70	0.17	8.34
	100	91.51 ± 7.86	-1.58	2.10
Bisoprolol	1	1.19 ± 0.13	4.77	9.57
	25	24.21 ± 3.07	-2.13	2.68
	100	101.01 ± 5.86	4.50	5.74
Diltiazem	5	4.70 ± 0.70	4.51	4.68
	100	109.97 ± 2.40	1.93	5.64
	600	631.98 ± 40.77	-3.95	2.64
Doxazosin	1	1.11 ± 0.07	10.74	6.68
	25	27.93 ± 1.46	3.47	5.52
	100	100.45 ± 2.86	0.50	0.61
Lisinopril	1	1.13 ± 0.10	13.00	9.01
	25	29.13 ± 2.38	3.46	6.71
	100	106.95 ± 5.34	-2.06	4.21
Losartan	25	23.90 ± 2.50	4.40	7.93
	250	259.25 ± 21.64	-0.47	2.85
	1000	1111.52 ± 33.24	1.66	0.91
Ramipril	1	1.12 ± 0.12	12.41	3.66
	25	21.33 ± 6.12	5.12	2.09
	100	94.96 ± 8.62	2.28	3.13
Simvastatin	1	1.20 ± 0.11	4.30	5.45
	25	23.62 ± 1.69	-0.89	3.00
	100	95.28 ± 9.53	-1.09	2.70
Valsartan	250	242.62 ± 24.98	-0.85	6.47
	2000	1972.39 ± 81.47	7.35	8.62
	4000	4021.61 ± 65.38	-3.20	4.35

Table 5.7 (b) Accuracy, precision and quantification of VAMS assay at the low, medium and high concentrations for each target drug after 8 weeks of storage at room temperature (n = 6).

Drug	Concentration in whole blood (ng/ml)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
Amlodipine	1	1.05 ± 0.09	4.89	8.17
	25	25.67 ± 2.85	2.69	11.09
	100	100.17 ± 11.29	0.17	11.27
Atenolol	50	55.71 ± 2.34	11.42	4.21
	500	523.31 ± 46.34	4.66	8.85
	1500	1516.29 ± 82.00	1.09	5.41
Atorvastatin	1	1.11 ± 0.06	11.13	5.35
	25	25.71 ± 2.18	2.83	8.49
	100	102.24 ± 13.62	2.24	13.32
Bisoprolol	1	1.10 ± 0.14	10.25	12.78
	25	25.55 ± 1.18	2.22	4.61
	100	97.26 ± 5.07	-2.74	5.21
Diltiazem	5	5.05 ± 0.52	1.03	10.32
	100	100.76 ± 8.33	0.76	8.27
	600	627.81 ± 49.60	4.63	7.90
Doxazosin	1	1.11 ± 0.11	10.78	10.11
	25	25.43 ± 3.49	1.73	13.72
	100	98.63 ± 11.48	-1.37	11.64
Lisinopril	1	0.99 ± 0.06	-1.01	6.41
	25	27.05 ± 3.66	8.19	13.55
	100	107.65 ± 10.07	7.65	9.36
Losartan	25	26.37 ± 2.82	5.48	10.70
	250	254.55 ± 22.11	1.82	8.69
	1000	977.61 ± 49.09	-2.24	12.18
Ramipril	1	1.08 ± 0.13	8.14	12.09
	25	24.07 ± 2.27	-3.70	9.44
	100	98.16 ± 10.37	-1.84	10.57
Simvastatin	1	1.08 ± 0.10	7.96	9.35
	25	25.35 ± 1.82	1.39	7.19
	100	94.20 ± 7.30	-5.80	7.75
Valsartan	250	253.57 ± 30.06	1.43	11.86
	2000	1921.63 ± 19.91	-3.92	1.04
	4000	4039.78 ± 173.14	5.99	8.80

5.2.9 Correlation studies between 903 sampling paper and VAMS extracts for the eleven target drugs

These studies were performed to get a feel of the relationship between analyte concentration from calibration samples prepared on 903 sampling paper and VAMS device. In the correlation studies, the ratios of analyte to internal standard concentrations of calibration standards (n = 6) for each target analyte on 903 sampling paper and VAMS device run on three separate days were compared by simple linear regression (Figure 5.3 (a) – (k)).

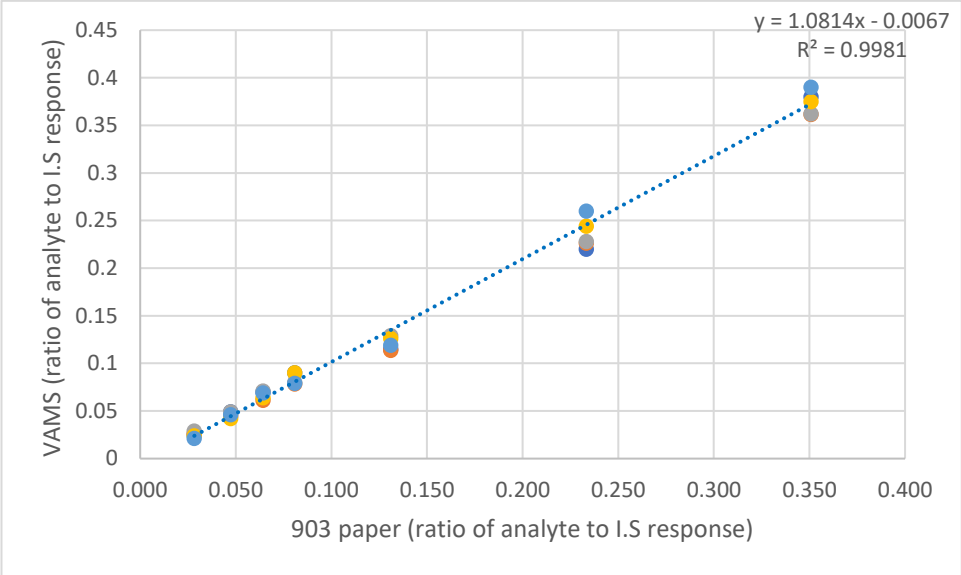


Figure 5.3 (a) Correlation plot of amlodipine calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

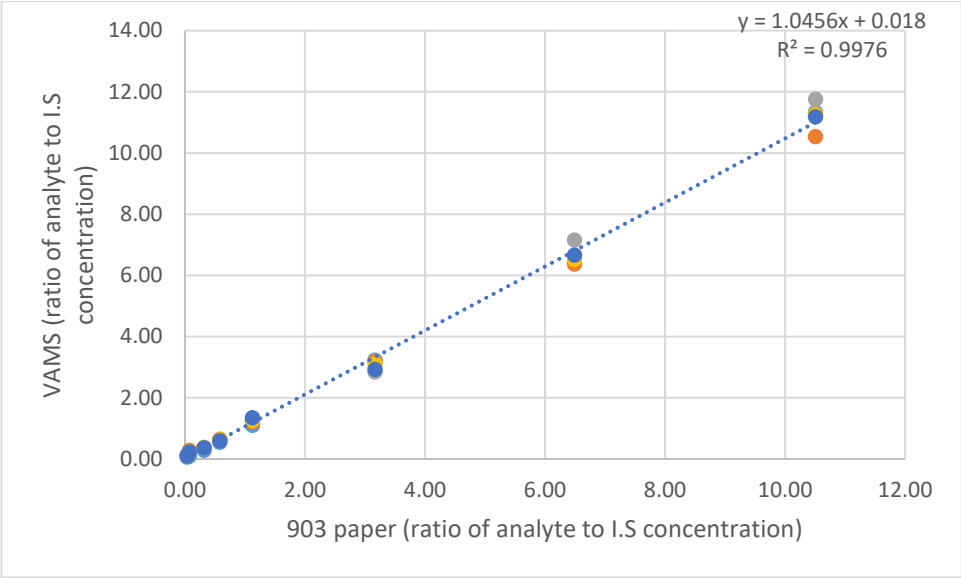


Figure 5.3 (b) Correlation plot of atenolol calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

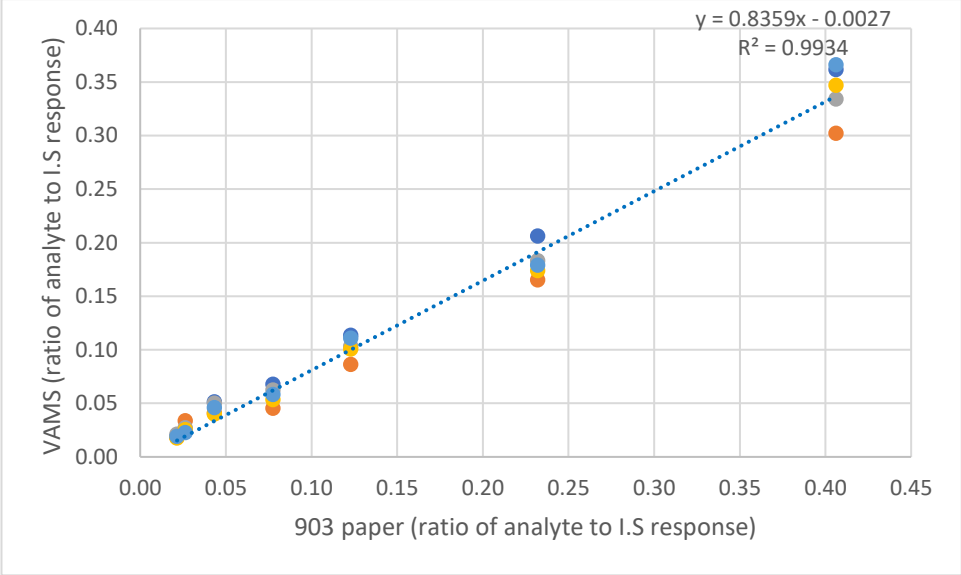


Figure 5.3 (c) Correlation plot of atorvastatin calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

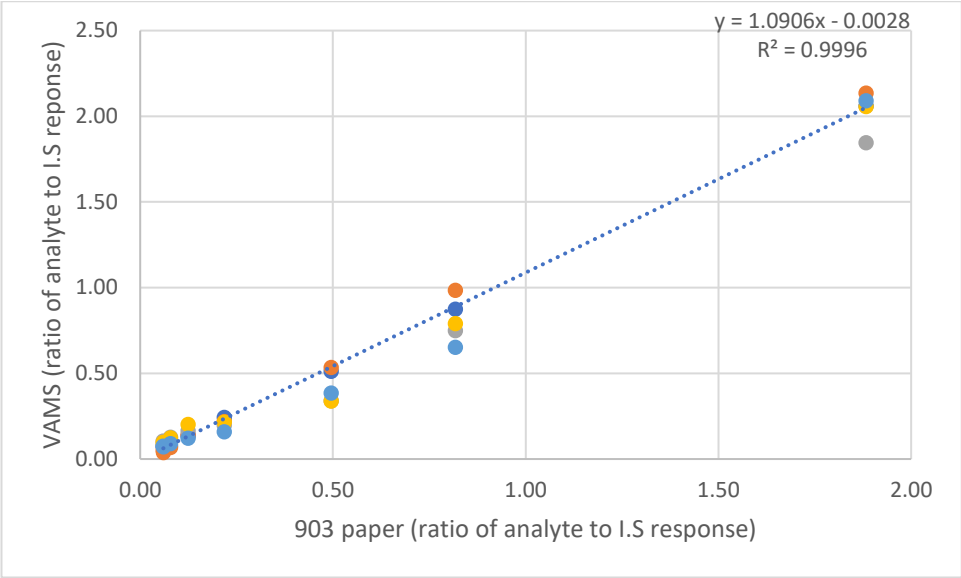


Figure 5.3 (d) Correlation plot of bisoprolol calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

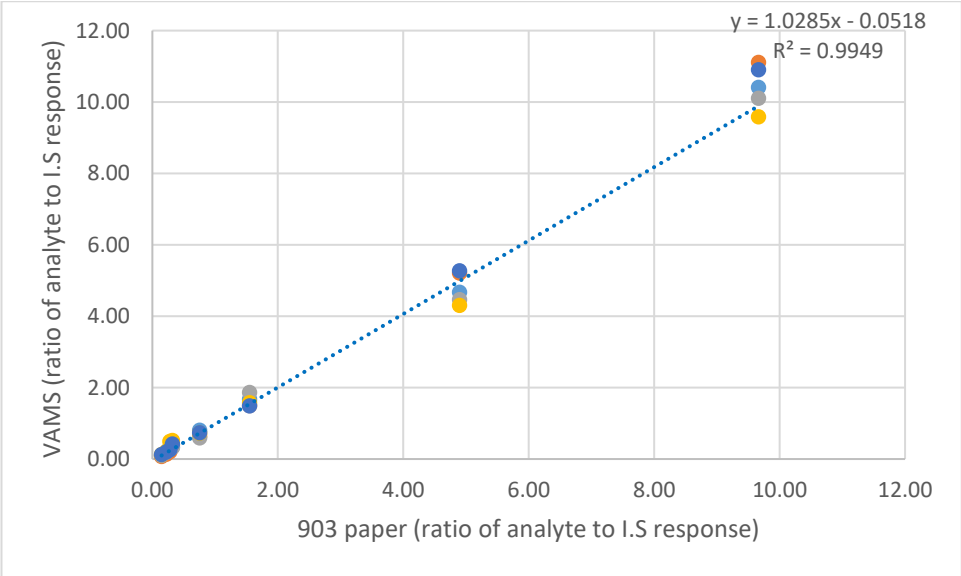


Figure 5.3 (e) Correlation plot of diltiazem calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

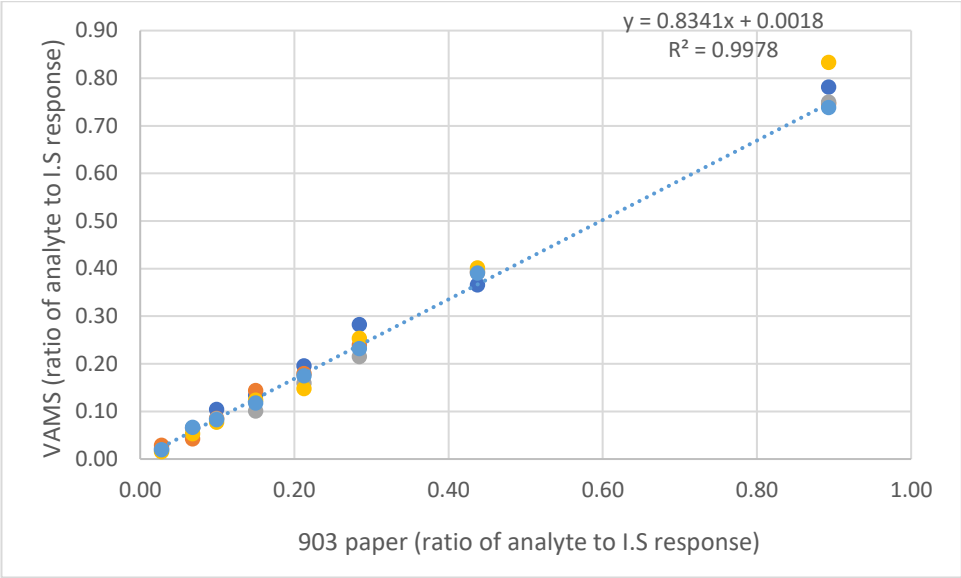


Figure 5.3 (f) Correlation plot of doxazosin calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

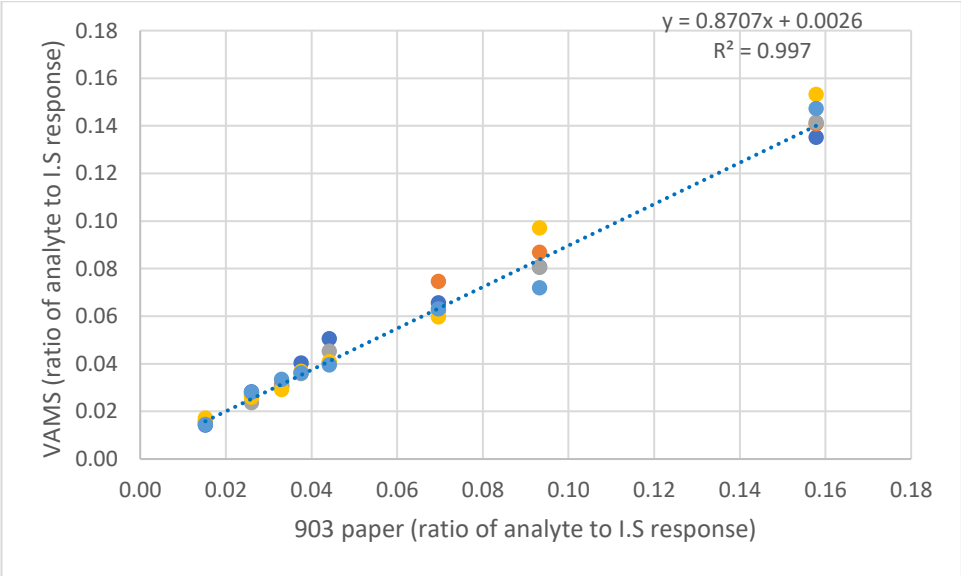


Figure 5.3 (g) Correlation plot of lisinopril calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

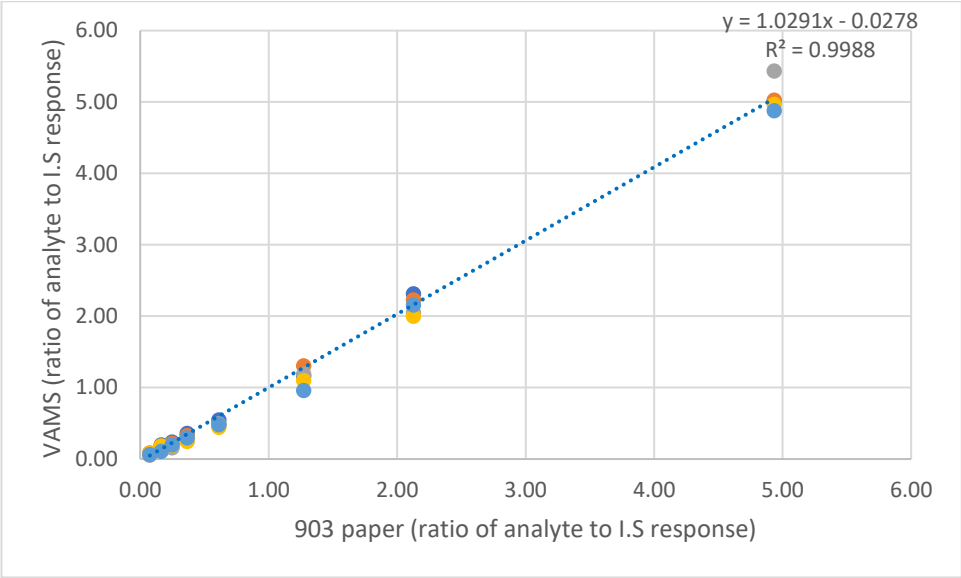


Figure 5.3 (h) Correlation plot of losartan calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

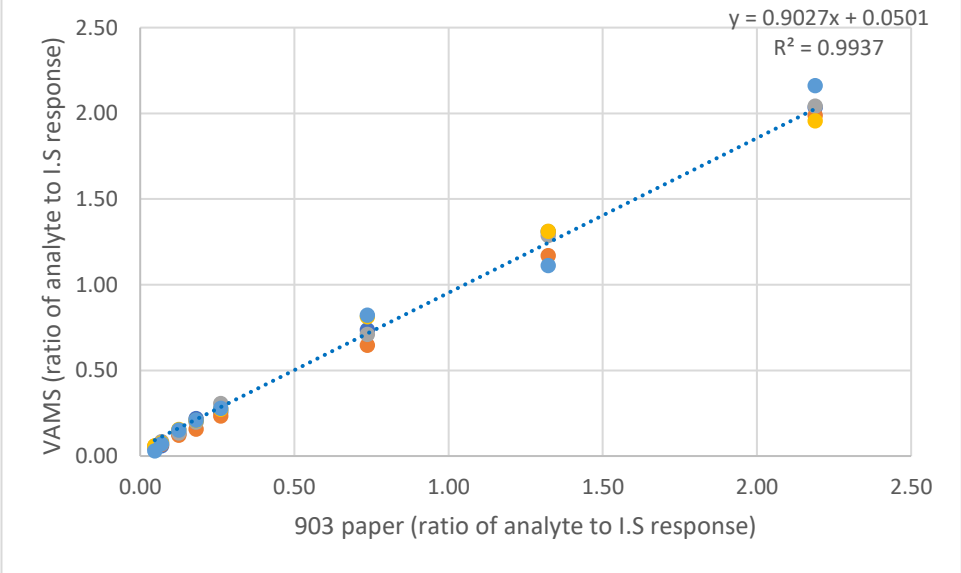


Figure 5.3 (i) Correlation plot of ramipril calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

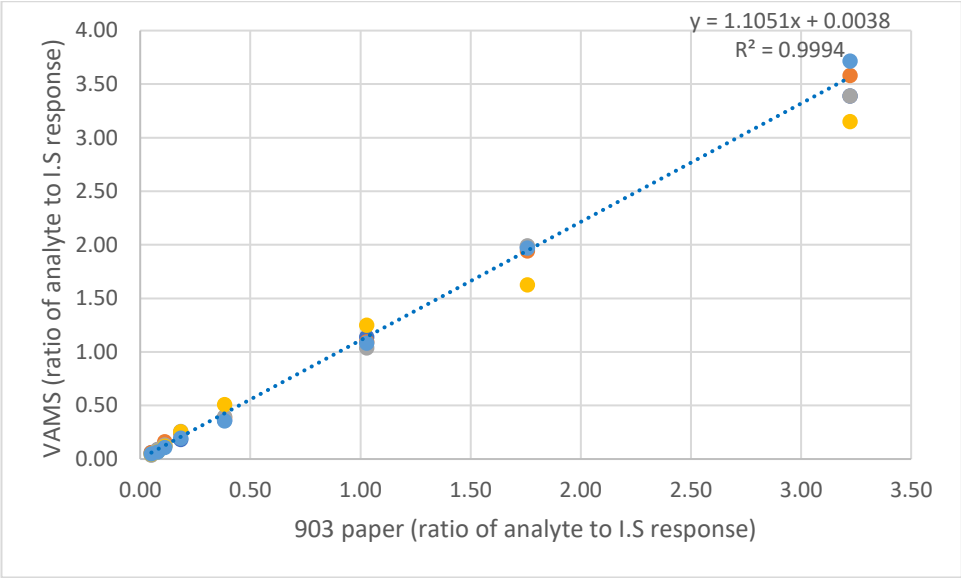


Figure 5.3 (j) Correlation plot of simvastatin calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

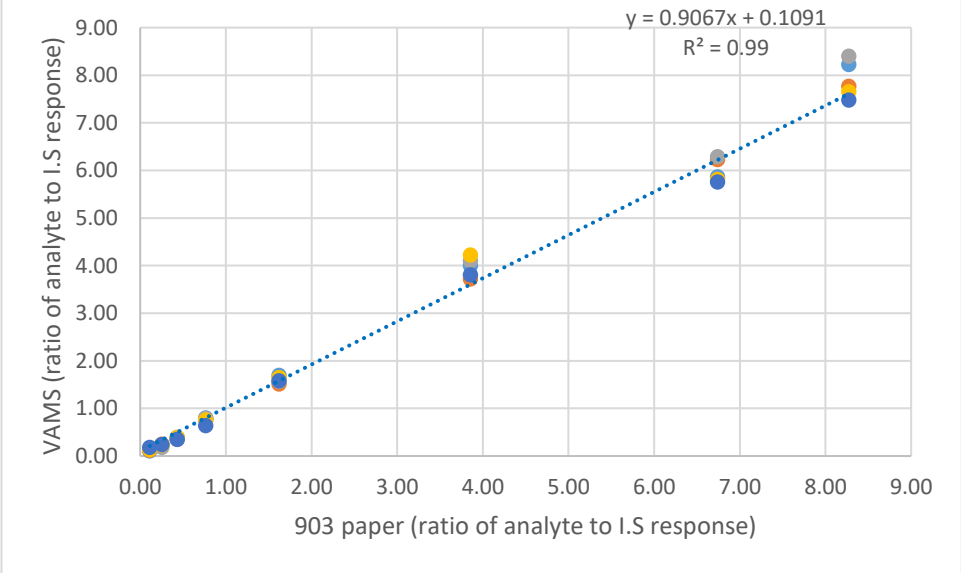


Figure 5.3 (k) Correlation plot of valsartan calibration standards obtained by analysis of extracts from 903 sampling paper (x) and VAMS device (y).

The linear regression equations from calibration standards (n = 6) run over three different days on 903 sampling paper and VAMS device showed good correlations between the ratios of analyte to internal standard response for all the CVD drugs (Figure 5.3 (a) – (k)). The correlation coefficient for the target drugs are: amlodipine R^2

= 0.998; atenolol R^2 = 0.997; atorvastatin R^2 = 0.993; bisoprolol R^2 = 0.999; diltiazem R^2 = 0.995; doxazosin R^2 = 0.998; lisinopril R^2 = 0.997; losartan R^2 = 0.998; ramipril R^2 = 0.994; simvastatin R^2 = 0.996; valsartan R^2 = 0.990. Correlations were assessed by calculating a linear regression line with 95% confidence intervals. The linear regression equations for 903 sampling paper extracts and VAMS extracts obtained in the correlation study (x = 903 sampling paper extracts, y = VAMS extracts) are shown in (Figure 5.3 (a) – (k)).

The results demonstrate that there is less variability in the ratio of analyte to I.S response data obtained by the analyses of the target cardiovascular drugs on 903 sampling paper and VAMS device.

5.3 Conclusion

Validation of the bioanalytical assay was successful on both the 903 sampling paper and VAMS device. Results for the various validation parameters demonstrated accuracy and precision values within acceptable range, except the hematocrit investigation. Results from the hematocrit investigation confirms that VAMS is independent of the Hct for all the investigated analytes. In contrast, results from 903 sampling paper shows that at extreme Hct values outside the normal range of 40 – 54% and 36 – 48% for healthy adult males and females, the volumetric hematocrit effect may be significant when a punch is used. Correlation analysis between 903 sampling paper and VAMS calibration standard extracts show that there is less variability in ratio of analyte to internal standard concentrations obtained by the two microsampling methods for the CVD drugs investigated. This indicates that both methods of microsampling can generate reliable quantitation data for the selected cardiovascular drugs studied in dried blood microsamples.

The overall validation results indicate that the developed assay was suitable for trials with volunteers. Thus, the validated bioanalytical method was applied to the quantification of the selected cardiovascular drugs in volunteer and patient dried blood samples collected on 903 sampling paper and VAMS.

Chapter 6 Application of validated LC-HRMS method for the analyses of volunteer blood samples collected on Whatman 903 cards and VAMS for the assessment of adherence to CVD prescription medication

This chapter discusses the application of the developed and validated 903 card and volumetric absorptive microsampling (VAMS) microsampling based LC-HRMS assay for the assessment of adherence to CVD prescription medication. 903 card and VAMS blood samples collected from two groups of participants (heart disease volunteers) in the UK and Iraq, prescribed with one or more of the CVD drugs: amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan were analysed with the developed LC-HRMS assay. The results of the analyses are also presented.

6.1 Introduction

Following method validation, application of the bioanalytical assay to clinical samples is key to demonstrate that the method can determine cardiovascular drug levels in volunteer samples. The two methods of microsampling (903 cards and VAMS) used for the validation of the developed assay in Chapter 5 were used for volunteer blood sample collection. It is however noteworthy that validation samples are fixed permanent “invitro” samples, which do not change over time and will therefore show less variability in results from the two microsampling methods as demonstrated by the correlation between 903 card and VAMS extracts from calibration standards in Chapter 5, section 5.2.9. However, this correlation may not remain constant for 903 card and VAMS extracts measured in volunteer samples. The reason being that volunteer samples are “invivo” samples, which is constantly changing due to metabolism of the drugs in the body. Hence sampling time delay between the collection of microvolume blood samples by a volunteer on 903 card and VAMs could influence this correlation. The volunteer samples were collected from two groups of participants (a) Heart disease volunteer samples from De Montfort University-UK referred to in this thesis as DMU samples and (b) Heart disease volunteer samples from the Al Sader Teaching

Hospital and the Misan Cardiac Centre, Iraq herein referred to as Iraq samples. The study has received ethical approval from the De Montfort University Research Ethics Committee. The inclusion criteria for volunteer selection was to include any volunteer prescribed with oral cardiovascular medication with or without other medications. As well as, volunteers who were not taking any medication at all to serve as control. The exclusion criteria was to exclude volunteers who were not on any prescribed cardiovascular medication. Using the power law for sample size calculation, with an 85% confidence level, the minimum number of volunteers required was 40.

6.2 Volunteer selection, DMU sample collection and storage on Whatman 903 cards and VAMS

One group of healthy volunteers prescribed with one or more of the selected target drugs were recruited from within De Montfort University staff. A dedicated webpage (DMU, 2016) about the research and its impact was created on the University's website to increase awareness of the study and to help recruit participants. Volunteers were issued with a participant information leaflet (PIL) (Appendix 2) to read prior to attending the sample collection in the laboratory. The PIL explained the purpose of the study, how participants will be involved and the contact details of the researchers in case of further questions. A consent form and mini questionnaire were then completed on arrival of the volunteer at laboratory (HB00.15) to confirm voluntary participation. The mini questionnaire was used to capture information on a participant's prescribed medicines, dosage, frequency of intake and approximate time since taking last doses in hours.

Self-collected volunteer blood samples were collected on the Whatman 903 cards and VAMS device by following the protocol documented in the participant information leaflet (Appendix 2). A series of blank control 903 card and VAMS samples were taken from some volunteers not prescribed any of the target drugs and used as control. Collected 903 card and VAMS samples were labelled in line with the ethics protocol and dried for at least 3 hours at room temperature before packing in sealed bags for secure storage in a locked cabinet.

6.3 Patient selection, Iraq sample collection and storage on Whatman 903 cards and VAMS

Cardiovascular disease patients attending a routine clinical follow-up at Al Sader Teaching Hospital and the Misan Cardiac Centre, Iraq were recruited for participation in the study. The PIL, consent form and mini questionnaire were translated into Arabic (Appendix 3) to help the patients understand the purpose of the study since English was not a first language. Each patient completed a consent form to confirm they understood the reason for the study and voluntarily agreed to participate before samples were collected. A mini questionnaire was also completed to collect information on a participant's prescribed medicines, dosage, frequency of intake and approximate time since taking last doses in hours. Since patients were attending routine follow up appointments, they had no prior knowledge of the research study until they attended the clinic. Both groups of study participants (volunteers in UK and patients in Iraq) were all prescribed with one or more of the target drugs amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan.

Patient blood samples were organised and collected from the Al Sader Teaching Hospital and the Misan Cardiac Centre, Iraq by another PhD student of the DBS Analysis research group in De Montfort University – UK by name Ahmed Alalaqi. A series of blank control 903 card and VAMS samples were taken from a second group of volunteers not prescribed any of the target drugs and used as control. The spot sizes were sufficient to allow the use of an 8mm punch on 903 sampling paper without compromising the DBS sample, samples of smaller spot sizes were rejected. For VAMS, the whole sample was used for extraction. The quality of the volunteer samples collected on 903 cards and VAMS were assessed. Eight spots on 903 cards were rejected for quantification because the blood sample was not sufficient to allow the use of an 8mm punch. In contrast to VAMS, only one sample was rejected due to incomplete filling.

6.3.1 Volunteer feedback on self-sample collection

903 cards require sufficient blood to be deposited within marked circles on the card. This made sampling difficult for some volunteers, as they had to produce sufficient blood from the lanced finger and direct the blood drop unto the card. In contrast, VAMS device is designed to wick the blood drop from the finger until the substrate is full seemingly making it easier to sample. Feedback on the ease of use of the two microsampling methods (Whatman 903 cards and VAMS) for blood sample collection was collected from DMU volunteer participants. Volunteer feedback centred on their experience on self-sampling with the two microsampling methods. Particularly concerning the ease of use of both the (903 card and VAMS) for blood sample collection. The feedback shows that self-sampled DBS or VAMS are a good alternative to venepuncture to use for the TDM of CVD drugs. Table 6.1 summarises the feedback on device usage received from volunteers who participated in the study.

Table 6.1 Summary of feedback provided by DMU volunteers on the ease of use of the two microsampling methods.

903 sampling paper	VAMS
• Difficult to drop the blood on the marked circles. • Last spot was small in size as bleeding stopped after the third spot. • Could not see the marked circles when dropping the spot on the card because I had to use the other hand to assist. • Spot dropped on the card before, finger could be directed unto the circle. • Took more time to sample on card • Will require assistance with card sampling. • Will take several practices to get samples in the marked circles. • Had to direct my finger to the marked circles, which was quite difficult to do. • Had to prick another finger to sample on card because did not bleed well. • A bit complex compared to VAMS.	• Sampling is easy as the device sucks the blood off the fingertip. • All four samplers were easily filled due to the small size of the tip • Prefer VAMS to card sampling, because it is so easy to use. • See VAMS as a very easy to use platform. • Was very quick self-sampling on VAMS. • Can easily do sampling on VAMS without assistance. • Will be willing to give sample again as it is easy. • Very easy to sample, no need to turn or direct finger after pricking. • Did not bleed well and could still fill the VAMS substrates. • VAMS is simple and quick to do.

6.4 Analyses of DMU samples collected on 903 card and VAMS by LC-HRMS

Acceptable samples on 903 card and VAMS were solvent extracted for analyses using the procedure described in section 4.5 of Chapter 4 to infer their adherence to prescription medication. The validated 903 sampling paper and VAMS based LC-HRMS method in Chapter 5 was successfully used for the identification and quantification of the target cardiovascular drugs in 240 Whatman 903 card and VAMS samples obtained from 41 volunteers. No false positive signals (Table 6.2) were detected from 903 card and VAMS samples from volunteers receiving no medication. The measured 903 card and VAMS drug concentrations obtained from DMU samples are presented in Table 6.2. The eclectic Cmax data from the literature for the individual drugs has also been included in Table 6.2 to provide reference values against which volunteer data can be compared. Values similar to, but lower than, the Cmax concentration depending on the time the dose was taken would be anticipated from volunteers who are adherent to prescribed medication. Likewise, absence of the drug in the volunteer's blood sample will indicate nonadherence. On this basis, the data in Table 6.2 would suggest that concern might be raised over the results from:

- volunteer 18 - where atenolol was detected but there was no detectable simvastatin
- volunteer 19 – no detectable ramipril signal
- volunteer 25 - no detectable atorvastatin signal but the anticipated lisinopril was detected

Table 6.2 903 sampling paper and VAMS concentrations of the studied cardiovascular drugs in DMU samples from volunteers prescribed with one or more of the CVD drugs investigated.

Sampling Device	N	Sex	CVD Drug	Time after Oral intake (h)	Conc (ng/ml) ±(s.d)	Cmax (ng/ml)
903	1	M	Bisoprolol 2mg	4	41.78 ± 1.99	37 - 87
VAMS			Bisoprolol 2mg	4	38.36 ± 2.55	
903			Doxazosin 4mg	4	32.74 ± 1.04	18 - 48
VAMS			Doxazosin 4mg	4	37.12 ± 4.38	
903			Valsartan 160mg	4	493.72 ± 8.78	879 - 3874
VAMS			Valsartan 160mg	4	503.11 ± 5.12	
903	2	M	Atorvastatin 10mg	11	8.88 ± 0.99	3.2 -10.5
VAMS			Atorvastatin 10mg	11	9.22 ± 1.49	
903			Losartan 50mg	11	28.95 ± 1.93	89 - 306
VAMS			Losartan 50mg	11	26.34 ± 2.08	
903	3	F	Losartan 75mg	22	20.60 ± 5.65	263 - 783
VAMS			Losartan 75mg	22	16.84 ± 3.92	
903	4	F	Simvastatin 20mg	13	2.90 ± 0.77	5.1 - 40.1
VAMS			Simvastatin 20mg	13	2.81 ± 0.30	
903	5	F	Ramipril 1.25mg	5	3.11 ± 0.37	11.1 - 31.1
VAMS			Ramipril 1.25mg	5	2.77 ± 0.24	
903	6	F	Losartan 100mg	5.5	11.60 ± 1.51	469 - 1131
903	7	M	Losartan 5mg	7	6.25 ± 3.41	89 -306
903	8	M	Atorvastatin (lowest)	16	6.11 ± 2.21	3.2 -10.5
VAMS			Atorvastatin (lowest)	16	4.98 ± 2.61	
903	9	F	Atorvastatin 20mg	17	6.77 ± 3.84	5.0 -20.5
VAMS			Atorvastatin 20mg	17	5.14 ± 2.20	
903	10	M	Ramipril 5mg	15	5.22 ± 0.31	11.1 - 31.1
VAMS			Ramipril 5mg	15	7.41±1.20	
903			Simvastatin 20mg	15	1.79±0.74	5.1 - 40.1
VAMS			Simvastatin 20mg	15	1.44±1.19	
903	11	M	Atorvastatin 10mg	14	5.21±1.99	3.2 -10.5
903	12	M	Bisoprolol 2mg	4	34.32±12.87	37 - 87
VAMS			Bisoprolol 2mg	4	32.93±1.96	
903			Doxazosin 4mg	4	32.40±2.13	18 - 48
VAMS			Doxazosin 4mg	4	37.21±3.58	
903			Valsartan 160 mg	4	407.16±14.73	879 - 3874
VAMS			Valsartan 160 mg	4	412.31±11.68	

Table 6.2 continued

Sampling Device	N	Sex	CVD Drug	Time after Oral intake (h)	Conc (ng/ml) ±(s.d)	Cmax (ng/ml)
903	13	M	Simvastatin	11	0.85±0.55	5.1 - 40.1
VAMS			Simvastatin	11	0.69±0.77	
903			Ramipril 10mg	2.5	9.37±1.04	11.1 - 31.1
VAMS			Ramipril 10mg	2.5	8.09±0.99	
903	14	F	Amlodipine 5mg	5	4.85±2.0	5.1 - 7.1
VAMS			Amlodipine 5mg	5	5.16±2.14	
903	15	M	Amlodipine 5mg	7	4.61±0.58	5.1 - 7.1
VAMS			Amlodipine 5mg	7	4.37±1.98	
903	16	F	Atorvastatin 10mg	17	2.86±1.72	3.2 -10.5
VAMS			Atorvastatin 10mg	17	2.02±0.93	
903			Losartan 100mg	7	65.48±3.72	469 - 1131
VAMS			Losartan 100mg	7	58.18±4.23	
903	17	F	Amlodipine 5mg	6	3.33±2.19	5.1 - 7.1
VAMS			Amlodipine 5mg	6	3.86±1.74	
903			Losartan 100mg	6	74.76±8.03	469 - 1131
VAMS			Losartan 100mg	6	81.55±5.13	
903	18	M	Atenolol 50mg	6	456.01±23.20	240 - 1370
VAMS			Atenolol 50mg	6	498.15±15.94	
903			Simvastatin 40mg	6	<LOQ	5.1 - 40.1
VAMS			Simvastatin 40mg	6	<LOQ	
903	19	F	Ramipril 10mg	18	<LOQ	11.1 - 31.1
VAMS			Ramipril 10mg	18	<LOQ	
903	20	M	Amlodipine 5mg	2	4.09±3.19	5.1 - 7.1
VAMS			Amlodipine 5mg	2	3.86±1.78	
903	21	F	Atorvastatin 20mg	14	14.01±2.39	5.0 -20.5
VAMS			Atorvastatin 20mg	14	10.88±2.37	
903			Bisoprolol 5mg	3	23.58±1.94	37 - 87
VAMS			Bisoprolol 5mg	3	21.88±7.27	
903	22	M	Lisinopril 20mg	?	37.02±8.59	50 - 88
VAMS			Lisinopril 20mg		29.59±5.31	
903	23	M	Amlodipine 10mg	4	8.24±2.00	11.7 - 14.1
VAMS			Amlodipine 10mg	4	7.99±3.18	
903			Ramipril 10mg	4	5.29±0.84	11.1 - 31.1
VAMS			Ramipril 10mg	4	4.26±0.69	
903			Simvastatin 20mg	10	1.32±0.42	5.1 - 40.1
VAMS			Simvastatin 20mg	10	1.29±0.86	

Table 6.2 continued

Sampling Device	N	Sex	CVD Drug	Time after Oral intake (h)	Conc (ng/ml) ±(s.d)	Cmax (ng/ml)
903	24	F	Amlodipine 5mg	2.5	5.11±1.34	5.1 - 7.1
VAMS			Amlodipine 5mg	2.5	4.49±2.33	
903			Ramipril 5mg	2.5	5.63±0.54	11.1 - 31.1
VAMS			Ramipril 5mg	2.5	4.82±0.39	
903	25	M	Atorvastatin 40mg	(not taking)	<LOQ	5.0 -20.5
VAMS			Atorvastatin 40mg		<LOQ	
903			Lisinopril 2.5mg	3.5	8.02±3.68	50 - 88
VAMS			Lisinopril 2.5mg	3.5	7.94±1.35	
903	26	F	Amlodipine 10mg	12	5.49±1.56	11.7 - 14.1
903			Losartan 12.5mg	12	37.57±2.54	43.6 - 125.4
903	27	M	Amlodipine 5mg	4	6.85±1.77	5.1 - 7.1
903	28	F	Bisoprolol 1.25mg	0.3	9.28±0.55	17 - 87
903	29	F	Ramipril 10mg	4 hrs	7.03±0.39	11.1 - 31.1
VAMS			Ramipril 10mg	4 hrs	7.68±2.47	
903	30	F	Ramipril 2.5 mg	3 hrs	6.49±0.96	<11.1 - 31.1
VAMS		F	Ramipril 2.5 mg	3 hrs	6.99±0.46	
903	31	F	Atorvastatin 40mg	15	18.36±7.20	>5.0 -20.5
VAMS		F	Atorvastatin 40mg	15	13.62±4.75	
903		F	Bisoprolol 5mg	8	24.46±5.70	37 - 87
VAMS		F	Bisoprolol 5mg	8	28.26±3.05	
903	32-36	F	None - Control	N/A	<LOQ	
VAMS	32-36		None - Control		<LOQ	
903	37-41	M	None - Control	N/A	<LOQ	
VAMS	37-41		None - Control		<LOQ	

Data from volunteer 18 raised concern initially because, information from the completed mini questionnaire by the volunteer revealed, both drugs were stated to have been taken at the same time whereas simvastatin should be taken in the evening. It may be that the patient was distracted and took two atenolol tablets rather than one of each tablet. This would lead to a measured atenolol levels corresponding to a 100mg dose as actually observed by the correlation between the measured

concentrations in DBS and VAMS and the Cmax data for a 100mg dose (Wu et al., 2003). Non-detectable simvastatin suggests that the patient was nonadherent bearing in mind that volunteers 4, 10 and 23 took simvastatin at a lower dose of 20mg and was still detected after 10 hours. Data from volunteer 19 showed no detectable level of ramipril, the prescribed drug but, according to the information provided on the mini questionnaire, the sample was collected 18 hours after the dose was taken. Since the LC-HRMS system operates in the full scan mode, the data was revisited to look for the metabolite of the drug (ramiprilat) at m/z 389.2071, but ramiprilat was also not detected. In this case the dose was 10mg and as can be seen for volunteer 10, prescribed a 5mg dose, levels of ramipril were detected 15 hours after taking a dose. This would suggest that volunteer 19 was not adherent to the treatment. It should be noted that pharmacogenetics effects may impact individual rate of drug metabolism leading to unexpected changes in drug levels in the blood (Joseph et al., 2014). Cambien et al (1994) demonstrated a significant link between angiotensin converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism and cardiovascular outcomes. However, the impact of this genetic polymorphism on ACE inhibitor response is not well understood. When asked about the data obtained volunteer 25 freely admitted not taking atorvastatin tablets for several days and was clearly nonadherent to the prescribed medication. The reason cited was adverse effects due to muscle pain and joint swelling. These results clearly indicate areas where a clinician would be unaware of an adverse clinical condition which they would be able to rectify to improve the individual's healthcare. This also demonstrates the robustness of the developed microsampling based LC-HRMS method.

The validated assay in Chapter 5 was also successfully used for the identification and quantification of the selected cardiovascular drugs in 552 VAMS and 903 card Iraq samples collected from 100 patients (Table 6.3). The expected drug(s) were detected for adherent patient samples after analysis. In cases where patient was prescribed with more than one class of medication, the assay correctly identified if the patient was not adherent to any of the medication. The assay also identified all medications for adherent patients taking more than one drug.

Table 6.3 903 card and VAMS concentrations of the studied cardiovascular drugs in Iraq samples from patients prescribed with one or more of the CVD drugs investigated.

Sampling device	N	CVD Drug	Time after Oral intake (h)	Concentration (ng/ml) (±sd)	Cmax (ng/ml)
903	1	Lisinopril 20mg	3	32.87±5.80	86.4 - 139
903	2	Simvastatin 20mg	10	<LOQ	5.0 - 40.0
903	3	Atenolol 50mg	36	<LOQ	240 - 1370
VAMS		Atenolol 50mg	36	<LOQ	
903	4	Bisoprolol 10mg	48	<LOQ	29 - 87
903	5	Valsartan 80mg	240	<LOQ	1010 - 2270
VAMS		Valsartan 80mg	240	<LOQ	
903	6	Valsartan 160mg	5	200.57±13.66	1930 - 4000
903	7	Atenolol 100mg	5	204.87±2.09	590 - 1370
VAMS		Atenolol 100mg	5	188.22±5.29	
903	8	Bisoprolol 5mg	10	11.28±0.69	29 - 87
VAMS		Bisoprolol 5mg	10	12.87±0.75	
903	9	Valsartan 160mg	12	135.80±3.35	1930 - 4000
903	10	Atorvastatin 20mg	48	<LOQ	3.2 - 10.6
VAMS		Atorvastatin 20mg	48	<LOQ	
903		Valsartan 40mg	12	147.34±2.42	535 - 1151
VAMS		Valsartan 40mg	12	160.21±5.11	
903	11	Bisoprolol 2.5mg	22	0.45±0.27	17.3 - 43.0
VAMS		Bisoprolol 2.5mg	22	0.34±0.14	
903	12	Simvastatin 20mg	12	<LOQ	5.0 - 40.0
903	13	Diltiazem 90mg	72	<LOQ	105.7 - 150.9
VAMS		Diltiazem 90mg	72	<LOQ	
903	14	Atenolol 50mg	9	200.11±7.75	240 - 1370
903	15	Valsartan 80mg	4	131.49±0.88	1010 - 2270
VAMS	15	Valsartan 80mg	4	126.45±2.62	
903	16	Losartan 25mg	10.5	19.04±3.66	43.6 - 125.4
VAMS		Losartan 25mg	10.5	17.23±6.75	

Table 6.3 continued

Sampling device	N	CVD Drug	Time after Oral intake (h)	Concentration (ng/ml) (±sd)	Cmax (ng/ml)
903	17	Losartan 100mg	13	112.30±1.60	469 - 1131
VAMS	17	Losartan 100mg	13	103.38±9.08	
903	17	Bisoprolol 5mg	13	75.96±3.12	29 - 87
VAMS	17	Bisoprolol 5mg	13	77.05±0.98	
903	18	Bisoprolol 2.5mg	3	42.60±4.82	17.3 - 43.0
	18	Valsartan 80mg	3	277.13±22.27	1010 - 2270
903	19	Valsartan 80mg	20	92.58±0.32	1010 - 2270
VAMS	19	Valsartan 80mg	20	86.09±0.88	
903	20	Lisinopril 10mg	8	54.65±5.92	41.8 - 80.5
VAMS	20	Lisinopril 10mg	8	48.69±3.30	
903	21	Losartan 50mg	48	<LOQ	89 - 306
VAMS	21	Losartan 50mg	48	<LOQ	
903	22	Atenolol 50mg	26	211.04±21.64	240 - 1370
903	23	Bisoprolol 5mg	3	5.74±0.70	29 - 87
VAMS	23	Bisoprolol 5mg	3	6.03±0.25	
903	23	Valsartan 160mg	32	<LOQ	1930 - 4000
VAMS	23	Valsartan 160mg	32	<LOQ	
903	24	Atenolol 50mg	27	86.11±2.05	240 - 1370
VAMS	24	Atenolol 50mg	27	79.18±4.61	
903	25	Bisoprolol 5mg	15	11.90±0.67	29.0 - 87.0
	25	Losartan 50mg	15	<LOQ	89 - 306
903	26	Atenolol 50mg	20	142.91±5.77	240 - 1370
VAMS	26	Atenolol 50mg	20	137.53±2.21	
903	27	Losartan 50mg	51	<LOQ	89.0 - 306.0
903	28	Atenolol 50mg	3.5	149.25±18.26	240 - 1370
VAMS	28	Atenolol 50mg	3.5	163.38±6.10	
903	29	Bisoprolol 5mg	21	5.13±0.20	29 - 87
VAMS	29	Bisoprolol 5mg	21	4.14±1.40	
903	29	Valsartan 80mg	36	<LOQ	1010 - 2270
VAMS	29	Valsartan 80mg	36	<LOQ	
903	30	Losartan 50mg	12	8.78±2.45	89 - 306

Table 6.3 continued

Sampling device	N	CVD Drug	Time after Oral intake (h)	Concentration (ng/ml) (±sd)	Cmax (ng/ml)
903	31	Atenolol 100mg	10	706.98±20.46	590 -1370
903	32	Atenolol 50mg	8	81.89±0.69	240 - 1370
VAMS	32	Atenolol 50mg	8	68.62±1.66	
903	33	Atenolol 100mg	13	248.30±9.12	590 - 1370
VAMS	33	Atenolol 100mg	13	254.16±5.37	
903	34	Atorvastatin 20mg	12	<LOQ	3.2 - 10.6
	34	Losartan 100mg	2	47.80±4.98	269 - 783
903	35	Atorvastatin 40mg	12.5	<LOQ	5.0 - 20.5
	35	Lisinopril 20mg	23.5	<LOQ	86.4 - 139
903	36	Bisoprolol 5mg	2.5	14.86±1.30	29 - 87
	36	Lisinopril 5mg	2.5	33.33±6.55	<41.8 - 80.5
903	37	Atenolol 25mg	15	94.17±8.36	<159 - 377
	37	Atorvastatin 20mg	15	<LLOQ	3.2 - 10.6
	37	Ramipril 5mg	15	3.75±0.22	9.3 - 20.8
903	38	Atorvastatin 20mg	76	<LOQ	3.2 - 10.6
VAMS	38	Atorvastatin 20mg	76	<LOQ	
903	38	Bisoprolol 5mg	76	<LOQ	29 - 87
VAMS	38	Bisoprolol 5mg	76	<LOQ	
903	39	Diltiazem 90mg	38	<LOQ	105.7 - 150.9
903	40	Diltiazem 60mg	1	25.17±0.57	21.9 - 86.7
VAMS	40	Diltiazem 60mg	1	29.51±2.20	
903	40	Losartan 50mg	13	<LOQ	89.1 - 306.1
VAMS	40	Losartan 50mg	13	<LOQ	
903	40	Valsartan 160mg	72	<LOQ	1930 - 4000
VAMS	40	Valsartan 160mg	72	<LOQ	
903	41	Atorvastatin 80mg	48	<LOQ	5.0 - 20.5
VAMS	41	Atorvastatin 80mg	48	<LOQ	
903	42	Atorvastatin 20mg	20.5	<LOQ	3.2 - 10.6
	42	Lisinopril 5mg	11	30.93±10.79	<41.8 - 80.5
903	43	Diltiazem 60mg	7.5	38.22±2.58	21.9 - 86.7
903	44	Lisinopril 10mg	13	38.61±6.16	41.8 - 80.5
VAMS	44	Lisinopril 10mg	13	36.33±3.48	

Table 6.3 continued

Sampling device	N	CVD Drug	Time after Oral intake (h)	Concentration (ng/ml) (±sd)	Cmax (ng/ml)
903	45	Atenolol 100mg	10	346.61±13.05	590 - 1370
VAMS	45	Atenolol 100mg	10	327.42±31.64	
903	46	Valsartan 80mg	11	115.93±2.12	1010 - 2270
VAMS	46	Valsartan 80mg	11	121.04±2.46	
903	47	Losartan 50mg	8	37.66±5.23	89.1 - 306.1
VAMS	47	Losartan 50mg	8	43.22±4.62	
903	48	Atorvastatin 40mg	12	14.04±1.28	5.0 - 20.5
VAMS	48	Atorvastatin 40mg	12	15.33±0.59	
903	48	Bisoprolol 5mg	2	42.72±3.15	29 - 87
VAMS	48	Bisoprolol 5mg	2	37.20±1.90	
903	49	Atorvastatin 20mg	32	<LOQ	3.2 - 10.6
903	49	Diltiazem 60mg	8.5	51.1±1.94	21.9 - 86.7
903	49	Valsartan 160mg	22.5	<LOQ	1930 - 4000
903	50	Valsartan 80mg	7.5	81.81±2.54	1010 - 2270
VAMS	50	Valsartan 80mg	7.5	90.16±3.27	
903	51	Valsartan 80mg	11	82.85±6.15	1010 - 2270
VAMS	51	Valsartan 80mg	11	78.24±4.48	
903	52	Atorvastatin 20mg	21.5	<LOQ	3.2 - 10.6
VAMS	52	Atorvastatin 20mg	21.5	<LOQ	
903	53	Diltiazem 90mg	11	87.26±6.9	105.7 - 150.9
VAMS	53	Diltiazem 90mg	11	79.1±11.5	
903	54	Lisinopril 10mg	10	33.30±6.57	41.8 - 80.5
VAMS	54	Lisinopril 10mg	10	36.90±3.51	
903	55	Valsartan 160mg	6	225.22±8.74	1930 - 4000
VAMS	55	Valsartan 160mg	6	206.57±6.53	
903	56	Valsartan 160mg	26	<LOQ	1930 - 4000
VAMS	56	Valsartan 160mg	26	<LOQ	
903	57	Atorvastatin 20mg	13	8.78±0.64	3.2 - 10.6
903	58	Valsartan 160mg	3.5	224.80±10.49	1930 - 4000
VAMS	58	Valsartan 160mg	3.5	231.15±6.36	
903	59	Diltiazem 60mg	18	8.86.7±0.34	21.9 - 86.7
VAMS	59	Diltiazem 60mg	18	10.8±0.81	

Table 6.3 continued

Sampling device	N	CVD Drug	Time after Oral intake (h)	Concentration (ng/ml) (±sd)	Cmax (ng/ml)
903	60	Bisoprolol 5mg	13	3.82±0.38	29 - 87
903	61	Atorvastatin 20mg	25.5	<LOQ	3.2 - 10.6
903	62	Valsartan 160mg	15.5	117.41±2.89	1930 - 4000
VAMS	62	Valsartan 160mg	15.5	120.74±3.85	
903	63	Bisoprolol 5mg	19	1.87±0.62	29 - 87
903	64	Bisoprolol 5mg	1	2.13±0.09	29 - 87
VAMS	64	Bisoprolol 5mg	1	2.46±0.18	
903	64	Lisinopril 10mg	1	24.97±2.05	41.75 - 80.47
VAMS	64	Lisinopril 10mg	1	28.23±2.13	
903	65	Lisinopril 5mg	10	45.86±2.87	<41.8 - 80.5
903	66	Bisoprolol 5mg	48	<LOQ	29 - 87
903	67	Bisoprolol 2.5mg	18	3.38±0.70	17 - 43
VAMS	68	Atenolol 100mg	2	781.05±48.83	590 - 1370
903	69	Bisoprolol 5mg	4.5	11.39±1.06	29 - 87
VAMS	69	Bisoprolol 5mg	4.5	10.33±3.88	
903	69	Valsartan 160mg	15	<LOQ	1930 - 4000
VAMS	69	Valsartan 160mg	15	<LOQ	
903	70 - 89	Controls	N/A	<LOQ	
VAMS	90 - 100	Controls	N/A	<LOQ	

In contrast with the data from DMU samples collected within the university staff (Table 6.2) where three volunteers representing 10% were suspected to be nonadherent, data from Iraq samples revealed 39% were suspected to be nonadherent (Table 6.3). The stated drug(s) were not detectable in twenty-seven (27) measured 903 card or VAMS samples (Volunteers 2, 3, 4, 5, 10, 12, 13, 21, 23, 25, 27, 29, 33, 34, 35, 37, 38, 39, 40, 41, 42, 49, 52, 56, 61, 66 and 69). Out of the patients who were suspected to be nonadherent, 63% had not taken their medication for more than 24 hours (Volunteers 3, 4, 5, 10, 13, 21, 23, 27, 29, 38, 39, 40, 41, 49, 56, 61 and 66). The huge difference in nonadherence between the two groups of participants (DMU samples and Iraq samples) could be due to the fact that the Iraq patients had no prior knowledge that

microvolume blood samples will be taking on the day of clinic visit to assess cardiovascular drug levels. Whereas volunteers recruited within DMU university staff were made aware of blood sample collection prior to sampling. Hence there is less possibility for the situation where a dose is taken because a test is anticipated (white coat syndrome) within the Iraq group.

Secondly, the cost of prescription treatment may be another factor likely to cause medication nonadherence in Iraq. Health care service in this part of the world is based on “cash and carry” where patients must pay for the service to see a doctor and afterwards pay for their treatment. Hence patients who cannot afford the medications will simply not buy them (Baroletti and Dell'Orfano, 2010; Baggarly et al., 2014). It is noteworthy that volunteers in the Iraq group had all seen a clinician on the day before giving a microvolume blood sample for analysis, yet some of them had still not taken either one or two of their medication. Figure 6.5 reveals that statins were the drugs with a high rate of nonadherence (83% for atorvastatin and 100% for simvastatin). Adverse effects may also be a factor for the high level of nonadherence in the Iraq group of volunteers. Cardiovascular disease patients are normally prescribed with medications from different therapeutic classes (complex regimen) (Anderson and Nawarskas, 2001). Combined therapy therefore increases the risk of adverse drug effects and drug interactions for cardiovascular disease patients. (Abolbashari et al., 2017). This is because the drugs may belong to different therapeutic classes and may have different physicochemical properties. Thus, the use of such an objective method of indicating adherence could help stimulate dialog between patients and clinicians to enable doctors to be aware and rectify the situation to improve the health of the patient.

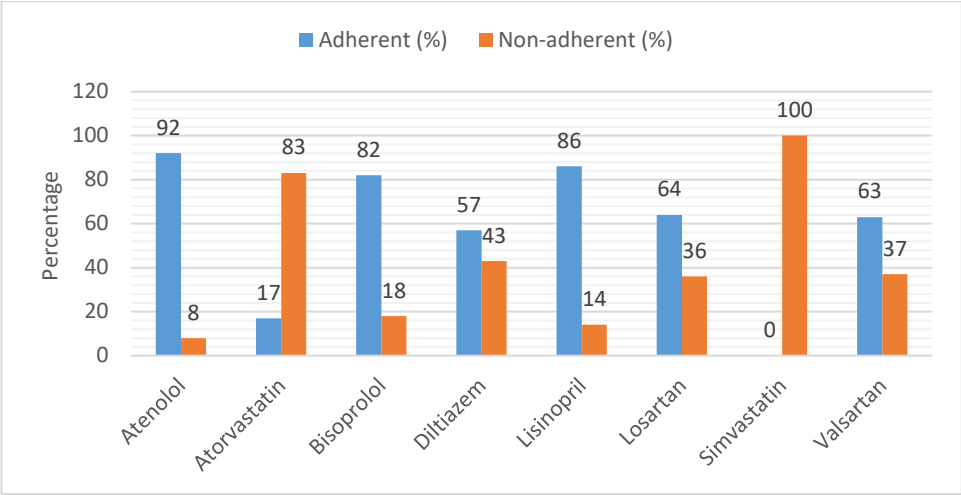


Figure 6.1 Compares the rate of adherence to nonadherence among the Iraq volunteer group for each of the target drugs.

The results obtained from the analyses of the two groups of volunteer samples show that monitoring of CVD drug concentrations in 903 card and VAMS samples is feasible and provides an objective means of inferring adherence to medication. The collected information will also be relevant to serve as the basis for clinical decision making. This will aid clinicians to personalise the treatment for each patient. This research has demonstrated the possible application of microsampling based LC-HRMS for TDM of CVD drugs in routine clinical settings. Such a convenient testing method will lead to improved adherence among CVD patients and allow for convenient self or at-home sampling so reducing required clinic visits. VAMS seems to be more promising than DBS in terms of ease of usage and most importantly overcomes issues like Hct bias and sample inhomogeneity. The only disadvantage of the VAMS microsampler is the relatively high cost compared to 903 cards. One VAMS sampler is about five times more expensive than a conventional DBS card which can collect five spots at a time. Hence cost may be a significant consideration for applicability in resource limited areas of the world (Kip et al., 2017).

When the results of this investigation are compared with that of published work utilising LC-MS/MS to test for therapeutic adherence among cardiovascular patient's in plasma or serum samples (Gonzalez et al., 2010; Gonzalez et al., 2011; Dias et al.,

2013; De Nicolo et al., 2016), the results obtained with the validated LC-HRMS assay demonstrates comparable sensitivity for the target drugs investigated. However, the novelty of applying blood microsampling methods over plasma/serum analysis in the TDM of cardiovascular drugs are: the uniqueness of self-use or "at home sampling" by the patient. This offers the convenience of assessment at any desired sampling time, which is otherwise not possible with plasma and serum sampling as the patient must travel to the clinic for blood sample to be taken. Secondly, the minimally invasive sampling procedure by means of a finger prick offers high patient acceptability, compared to venepuncture, which is highly invasive when plasma or serum is used for analysis. Thirdly, the amount of blood sample (microvolume) needed for analyses (≤30µl) which makes it ideal for routine clinical testing, in contrast with (~5ml) of blood required at each sampling time for plasma and serum analyses.

When the results of this investigation are compared with published work utilising urine analysis which only serve as a qualitative tool to assess medication adherence (Tomaszewski et al., 2014; Hamdidouche et al., 2015; Lawson et al., 2016; De Nicole et al., 2017, Hamdidouche et al., 2017), the results obtained with the validated LC-HRMS assay provides quantitative (confirmatory) data based on patient's blood drug levels which will be more useful to a clinician from a treatment point of view. This is because blood drug concentration is related to the effectiveness of the treatment. Hence it will help the clinician to tailor the treatment to each patient based on their individual differences in drug metabolism. The novelty of applying blood microsampling methods over urine analyses in the TDM of cardiovascular drugs are: The ability to generate information on the drug levels in the patient's blood, which is key to relate adherence to positive clinical outcomes. This is not possible when urine samples are used for analyses since the relationship between the time of ingestion, the dose taken, and the levels of drug in blood cannot be established. Secondly, quantitative blood concentration data provides vital information on drug levels for each patient enabling treatment to be tailored or personalised, which is not possible when urine samples are used. A major limitation of assessing medication adherence by direct methods is the white coat syndrome, where patients take the dose before clinic visits. This is reported

to be very common when urine samples are used for analysis (MacLaughlin et al., 2005). However, the developed microsampling based LC-HRMS assay/approach to inferring adherence can identify the situation where a dose is taken because a test is anticipated. This is comparable to a single dose trial and the pharmacokinetics would lead to a rapid increase followed by a decrease in the drug concentration in the blood depending on the $t_{1/2}$ of the drug, rather than a steady state situation (Chapter 1, Figure 1.1). Analyses of two 903 card or VAMS samples collected several hours apart, from the same volunteer, would clarify the situation. A drug concentration significantly less in the second sample would indicate that the dose was taken in anticipation of the test whereas a comparable level is indicative of a steady state because of adherence to prescription.

Lastly, except for Hamdidouche et al (2017) who reported a total analysis time of 5 minutes per sample, all the previously cited work on plasma and urine LC-MS/MS assay for the assessment of cardiovascular medication adherence reported, a total analysis time of between 10 to 48 minutes per sample. Such long analyses time makes these assays unfeasible to use in clinical and hospital settings since there may be numerous samples to run. In contrast, the microsampling based LC-HRMS assay has a run time of just 2.5 minutes making it feasible for high throughput robust screening in clinical and hospital settings.

The main limitation of the developed microsampling based LC-HRMS assay in this study, is the requirement of a separate extraction procedure for amlodipine, which therefore means that four DBS or VAMS samples are required for the analyses of volunteer dried blood samples where volunteers are prescribed with amlodipine in addition to any of the other 10 (atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, valsartan and simvastatin) CVD drugs.

Chapter 7 Innovations in microsampling methods

This chapter gives an overview of the current new developments in blood microsampling methods introduced to resolve the problems of hematocrit (Hct) effect and volumetric inaccuracies observed when the traditional DBS card is used for blood sample collection followed by the analysis and quantification of therapeutic drugs in humans.

7.1 Introduction

Microsampling is the only sampling procedure with the possibility to make self-sampling or “at home use” a reality in clinical diagnostics and other life science applications. However, the hurdles surrounding volumetric inaccuracies of blood sample from different individuals’ due to the Hct effect presented by using the conventional DBS card has hampered its acceptance in regulated bioanalysis. These hurdles have been overcome by the development of microsampling devices designed to resolve the challenges with conventional DBS card usage (Deniff and Spooner, 2014; Sturm et al., 2015; Verhaeghe et al., 2017). Examples of such devices are VAMS, Noviplex plasma preparation cards, Ahlstrom 167L cards, Hemaxis – DB blood collection device, Tomtec dry media spot slides and the HemaPen, which are discussed below.

7.1.1 HemaSpot-HF device

HemaSpot-HF device is an easy to use microsampler for blood sampling and robust storage/shipping of blood samples at ambient temperatures. HemaSpot-HF uses a finger stick to collect and dry blood within a secure cartridge. The sample can be safely and easily shipped to the laboratory for analysis.

HemaSpot-HF device consists of a robust cartridge housing a fan shaped absorbent paper known as the HemaForm (Figure 7.1). The HemaForm consist of eight identical strips surrounded by a desiccant and covered with a rubber application surface that has a small opening to allow entry for blood. After lancing the finger, three drops of

blood are deposited onto the small opening, which allows the blood sample to be absorbed by the HemaForm. Collected samples on the HemaSpot-HF device is left for two minutes and then secured by the tamper resistant latches on the edge of the cartridge to prevent contamination. The device can be shipped immediately at ambient temperature to the laboratory because, the desiccant surrounding the HemaForm completes the drying of the sample making the secured cartridge moisture proof. This contrasts with a conventional DBS sampling where collected samples must be dried for at least two hours prior to shipping.

The HemaSpot-HF device comes with a printed bar code on the cartridge which can be scanned or read by standard readers such as smart phones or laboratory scanners for sample identification and labelling. The secure cartridge is opened with a special tool at the laboratory and part of the HemaForm removed with tweezers for analysis. The remaining dried blood samples can be stored securely in the moisture-tight cartridge. Since the device does not sample accurate volumes, it is not known if the device is able to eliminate the analytical bias caused by the Hct (Henion et al., 2013).

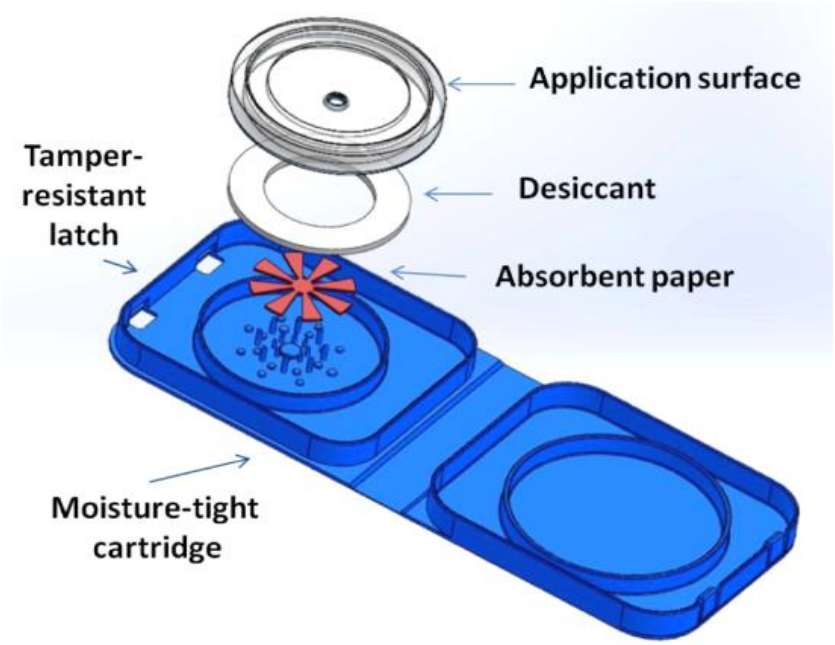


Figure 7.1 A schematic of a HemaSpot - HF device (Spot On Science, TX, USA, 2013).

7.1.1.1 Comparison of ratios of analyte to internal standard response for the CVD drugs on the HemaSpot – HF device and 903 sampling paper

The analytical performance of the HemaSpot – HF device was compared to the traditional 903 sampling paper by investigating the ratio of analyte to internal standard (IS) response for atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan extracted from the HemaSpot – HF device and the 903 sampling paper as control. DBS calibration standards, were prepared at the low, medium and high concentration of each target drug using the protocol described in Chapter 4, section 4.3.2.

7.1.1.2. Spotting of prepared blood standards on HemaSpot-HF device and 903 sampling paper.

3x30µl (90µl) volume of each of the prepared blood standards (blank, low, medium and high) were spotted through the sampling entrance in the clear transparent cover unto the applicator disk of the HemaSpot - HF Device. The blood was allowed to soak completely to the edges of the fan form. Blood standards were spotted on 903 sampling paper as described by the protocol in Appendix 1. Samples were air dried for at least 3 hours at room temperature. The dried samples were stored in number or code labelled re-sealable polythene bags in a secure cabinet in Lab HB 00.15.

7.1.1.3. Extraction of target analytes from the HemaSpot – HF device and 903 sampling paper

Two (2) fingers containing (~20µl) of blood sample were cut from the centre of the prepared HemaSpot-HF blood sample. For the prepared standards on 903 sampling paper, an 8mm disk (~20µl) was punched from the centre of the spot. Samples were transferred into a clean 1.5ml microcentrifuge tube and extracted using the procedure described in Chapter 4, section 4.5.1. Table 7.1 shows the ratio of analyte to internal standard data for the 2 microsampling methods investigated.

Table 7.1 (a) – (j) The ratio of analyte to internal standard response for the HemaSpot-HF and 903 sampling paper at the low, medium and high concentration levels of the 10 CVD drugs (n = 6).

(a) Atenolol

Nominal conc. (ng/ml)	50		500		1500	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.42	0.38	3.18	2.75	6.99	6.27
SD	0.02	0.02	0.23	0.18	0.43	1.24
Precision (CV%)	3.94	5.16	4.40	3.10	2.39	6.46

(b) Atorvastatin

Nominal conc. (ng/ml)	1		25		100	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.03	0.03	0.09	0.09	0.27	0.29
SD	0.003	0.005	0.01	0.01	0.02	0.04
Precision (CV%)	10.13	11.76	7.37	5.48	8.87	14.07

(c) Bisoprolol

Nominal conc. (ng/ml)	1		25		100	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.05	0.08	0.54	0.66	2.46	2.66
SD	0.01	0.01	0.03	0.08	0.16	0.27
Precision (CV%)	13.89	9.14	5.47	11.93	6.32	7.50

(d) Diltiazem

Nominal conc. (ng/ml)	5		100		600	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.38	0.43	1.55	1.34	9.31	9.05
SD	0.01	0.02	0.15	0.17	0.28	0.40
Precision (CV%)	3.77	4.49	8.04	6.01	1.29	4.03

(e) Doxazosin

Nominal conc. (ng/ml)	1		25		100	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.13	0.15	0.33	0.34	0.84	0.87
SD	0.02	0.01	0.01	0.01	0.03	0.05
Precision (CV%)	13.53	10.23	2.75	3.89	3.62	5.52

(f) Lisinopril

Nominal conc. (ng/ml)	1		25		100	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.03	0.03	0.06	0.07	0.13	0.15
SD	0.008	0.002	0.00	0.01	0.01	0.01
Precision (CV%)	11.13	6.68	7.11	12.70	6.91	4.43

Table 7.1 continued

(g) Losartan

Nominal conc. (ng/ml)	25		250		1000	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.17	0.20	1.00	1.06	5.80	6.32
SD	0.01	0.02	0.07	0.14	0.11	0.50
Precision (CV%)	6.50	10.61	7.03	13.03	1.95	7.92

(h) Ramipril

Nominal conc. (ng/ml)	1		25		100	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.08	0.06	0.44	0.43	1.46	1.32
SD	0.04	0.06	0.11	0.04	0.06	0.23
Precision (CV%)	9.46	8.40	9.27	3.59	2.57	8.91

(i) Simvastatin

Nominal conc. (ng/ml)	1		25		100	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.09	0.08	0.42	0.61	3.45	2.74
SD	0.01	0.02	0.05	0.06	0.11	0.38
Precision (CV%)	10.30	9.25	13.41	9.86	7.59	13.78

(j) Valsartan

Nominal conc. (ng/ml)	250		2000		4000	
Sampling method	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**	**903 paper**	**HemaSpot**
Ratio of Analyte to I.S	0.25	0.23	2.01	1.73	6.09	5.81
SD	0.02	0.03	0.13	0.29	0.89	0.78
Precision (CV%)	6.89	11.47	6.58	11.79	14.55	13.51

Sampling of prepared blood calibration standards on the HemaSpot – HF device, took a longer time (~4 minutes) to complete compared to using the 903 sampling paper which was completed in one minute. This may be due to the diameter of the application surface (sample entrance) in the clear transparent cover being very small. Hence, the blood drop collects as a bulb at the entrance of the device and takes time to soak through the absorbent paper, especially with the second and third drops of blood. Another observation was that, even though the application surface of the device is perforated, two additional perforations had to be made on the application surface with a clean needle to aid suction of the blood into the absorbent paper and to prevent bubble formation at the sampling entrance.

The ratio of analyte to IS concentration data for atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan extracted from the HemaSpot – HF device and 903 sampling paper were comparable at the low, medium and high concentrations of the target drugs investigated (Table 7.1). No marked difference was observed between data for the two microsampling methods. This effect could likely be due to the nature of the sampling materials. HemaSpot – HF device and 903 sampling paper are made of cellulose based paper and may therefore have similar physical and chemical properties (Koster et al., 2015). Hence the observed similarity in data acquired. An overlaid total ion chromatogram (TIC) profile for a medium calibration standard on the HemaSpot – HF device and 903 sampling paper (Figure 7.2), shows that there is no difference between the two sampling materials.

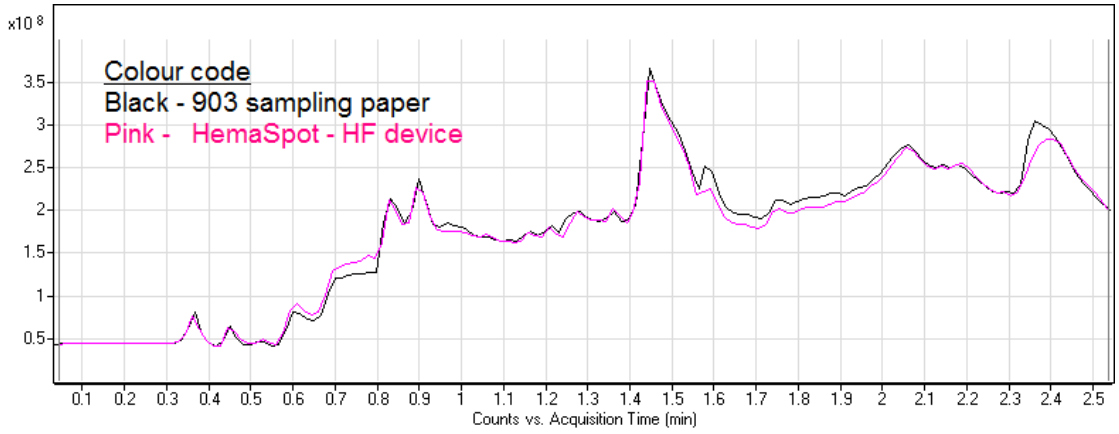


Figure 7.2 Overlaid TIC of a medium blood calibration standard extract on HemaSpot – HF device and 903 sampling paper.

7.1.2 TOMTEC Dry Media Spot Slides

The Tomtec dry media spot slides are pre-cut DBS cards that comes in various spot sizes of 6mm, 8mm and 10mm to enable integration into a 96 well, 48 well and 24 well formats depending on application. The sampling device is made of two media, either cellulose (PDMS 4) or polyester polymer (PDMS 7) material (Figure 7.3). Both devices are designed to work with automated systems developed by the manufacturer. Polyester does not allow blood to soak into the fibres, hence the blood sample is housed by the interstices between the fibres. The dry media spot slides utilise fixed

volume sampling by means of a capillary which is used to collect the blood sample and then deposited onto the card. The idea is to reduce or eliminate the Hct bias caused by using a fixed punch from a DBS sample. The dry media spot slides are pre-etched (Figure 7.3) to enable whole sample analysis rather than punch a fixed size after sampling.

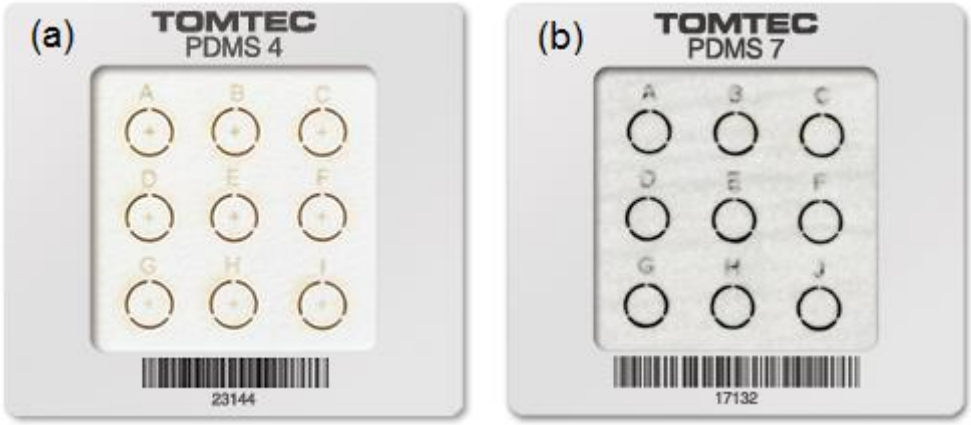


Figure 7.3 A picture of (a) Tomtec PDMS 4 and (b) the PDMS 7 dry media spot slides (Tomtec, 2015).

7.1.2.1. Evaluation of extraction efficiency of the selected CVD drugs on Tomtec PDMS 4, PDMS 7 and 903 sampling paper

The extraction efficiency of atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan were evaluated on the Tomtec PDMS 4 and PDMS 7 using 903 sampling paper as the control. DBS calibration standards were prepared at the blank, low, medium and high concentrations for the target CVD drugs (Chapter 4, Table 4.7). 10µl volume of each prepared standard was spotted unto 903 sampling paper, Tomtec PDMS 4 and Tomtec PDMS 7 sampling cards. The whole spot was used for extraction following the procedure described in Chapter 4, section 4.5.1. Extracts were analysed using the developed and validated LC-HRMS assay in Chapter 5. Extraction efficiency was determined using the procedure described in Chapter 5, section 5.2.5. Table 7.2 shows the extraction efficiency of the selected CVD drugs at the low, medium and high concentrations investigated for 903 sampling paper, Tomtec PDMS 4 and Tomtec PDMS 7 sampling cards.

Table 7.2 Extraction efficiency data for 903 paper, Tomtec PDMS 4 and PDMS 7 sampling materials at the low, medium and high concentration ranges of the target CVD drugs.

(a) Atenolol

Sampling method	Nominal concentration (ng/ml)	50	500	1500
903 paper	Recovery (%)	89.51	85.26	89.21
	SD	13.81	7.04	1.66
	Precision (CV %)	12.61	7.39	1.68
Tomtec PDMS 4	Recovery (%)	80.35	85.75	84.71
	SD	6.38	5.64	2.38
	Precision (CV %)	7.06	5.89	2.51
Tomtec PDMS 7	Recovery (%)	37.94	50.18	51.40
	SD	3.31	4.60	1.43
	Precision (CV %)	8.72	9.18	2.01

(b)Atorvastatin

Sampling method	Nominal concentration (ng/ml)	1	25	100
903 paper	Recovery (%)	98.34	102.87	101.79
	SD	9.92	7.83	7.13
	Precision (CV %)	9.47	13.42	6.38
Tomtec PDMS 4	Recovery (%)	98.86	94.33	97.10
	SD	5.58	2.48	6.04
	Precision (CV %)	14.67	12.46	6.22
Tomtec PDMS 7	Recovery (%)	180.09	192.45	132.69
	SD	39.22	45.72	8.94
	Precision (CV %)	13.43	23.75	6.74

(c) Bisoprolol

Sampling method	Nominal concentration (ng/ml)	1	25	100
903 paper	Recovery (%)	88.68	86.64	80.04
	SD	9.48	3.17	5.27
	Precision (CV %)	6.84	5.59	8.78
Tomtec PDMS 4	Recovery (%)	77.65	75.98	62.26
	SD	3.38	7.53	1.35
	Precision (CV %)	3.14	13.45	2.59
Tomtec PDMS 7	Recovery (%)	136.73	73.15	38.60
	SD	30.42	26.59	3.29
	Precision (CV %)	3.25	36.35	8.53

(d)Diltiazem

Sampling method	Nominal concentration (ng/ml)	5	100	600
903 paper	Recovery (%)	86.74	74.70	93.00
	SD	15.61	3.97	1.28
	Precision (CV %)	12.32	11.43	1.75
Tomtec PDMS 4	Recovery (%)	75.89	75.01	89.92
	SD	19.23	4.40	0.94
	Precision (CV %)	14.15	9.78	1.35
Tomtec PDMS 7	Recovery (%)	25.13	20.09	12.86
	SD	7.84	5.58	0.91
	Precision (CV %)	4.02	27.78	7.08

Table 7.2 continued

(e) Doxazosin

Sampling method	Nominal concentration (ng/ml)	1	25	100
903 paper	Recovery (%)	94.43	87.99	83.62
	SD	6.43	3.55	4.57
	Precision (CV %)	5.47	4.04	6.89
Tomtec PDMS 4	Recovery (%)	86.44	93.83	91.21
	SD	6.74	3.79	4.73
	Precision (CV %)	6.65	4.04	7.74
Tomtec PDMS 7	Recovery (%)	30.18	22.36	14.73
	SD	11.95	3.34	0.84
	Precision (CV %)	7.46	14.93	5.71

(f) Lisinopril

Sampling method	Nominal concentration (ng/ml)	1	25	100
903 paper	Recovery (%)	102.11	96.30	98.01
	SD	6.85	11.85	7.94
	Precision (CV %)	5.51	10.18	7.35
Tomtec PDMS 4	Recovery (%)	105.49	102.24	90.08
	SD	18.14	14.14	7.27
	Precision (CV %)	7.10	11.94	5.59
Tomtec PDMS 7	Recovery (%)	162.98	197.68	127.78
	SD	34.66	24.37	6.08
	Precision (CV %)	13.18	12.33	4.76

(g) Losartan

Sampling method	Nominal concentration (ng/ml)	25	250	1000
903 paper	Recovery (%)	102.06	102.15	105.32
	SD	2.78	12.58	4.75
	Precision (CV %)	2.73	12.34	4.51
Tomtec PDMS 4	Recovery (%)	102.13	101.41	91.97
	SD	10.68	11.05	1.07
	Precision (CV %)	10.45	10.90	1.16
Tomtec PDMS 7	Recovery (%)	99.54	89.00	79.26
	SD	5.30	11.08	5.17
	Precision (CV %)	5.07	12.45	6.52

(h) Ramipril

Sampling method	Nominal concentration (ng/ml)	1	25	100
903 paper	Recovery (%)	104.49	100.65	100.69
	SD	11.358	8.405	2.032
	Precision (CV %)	10.871	8.351	2.018
Tomtec PDMS 4	Recovery (%)	98.01	102.07	95.46
	SD	10.93	9.07	3.24
	Precision (CV %)	11.15	8.55	3.39
Tomtec PDMS 7	Recovery (%)	64.87	60.39	51.74
	SD	6.28	8.05	2.53
	Precision (CV %)	8.39	13.33	4.89

Table 7.2 continued

(i) Simvastatin

Sampling method	Nominal concentration (ng/ml)	1	25	100
903 paper	Recovery (%)	66.22	62.32	59.18
	SD	15.99	6.01	3.53
	Precision (CV %)	7.40	12.44	6.51
Tomtec PDMS 4	Recovery (%)	67.18	73.53	51.85
	SD	6.36	13.30	2.03
	Precision (CV %)	4.05	14.22	4.24
Tomtec PDMS 7	Recovery (%)	32.94	13.38	7.31
	SD	6.08	0.94	0.41
	Precision (CV %)	9.66	7.04	5.64

(j) Valsartan

Sampling method	Nominal concentration (ng/ml)	250	2000	4000
903 paper	Recovery (%)	91.83	89.17	94.37
	SD	4.24	4.08	5.15
	Precision (CV %)	4.61	6.89	10.14
Tomtec PDMS 4	Recovery (%)	72.89	70.91	64.62
	SD	2.21	10.50	0.84
	Precision (CV %)	4.18	14.80	1.89
Tomtec PDMS 7	Recovery (%)	98.06	94.94	75.11
	SD	9.40	4.62	6.78
	Precision (CV %)	6.35	5.93	9.03

The results on extraction efficiency (recovery) show consistent, reproducible and comparable data for all target drugs on Tomtec PDMS 4 (cellulose based material) and 903 sampling paper. This is expected because, Tomtec PDMS 4 and 903 sampling paper are made of cellulose and thus may not show any wide differences in physical and chemical properties. In contrast with extraction efficiency data on Tomtec PDMS 7 (polyester sampling cards) drug recoveries of the target analytes were very poor for all target drugs (<50%), except for losartan and valsartan where mean recoveries were ~89%.

The results on Tomtec PDMS 7 suggest significant matrix effects because of co-eluting compounds from the polyester sampling material resulting in ion suppression for target drugs (atenolol, bisoprolol, diltiazem, doxazosin, ramipril and simvastatin) and signal enhancement for (atorvastatin and lisinopril). Polyester is a class of polymer consisting of several monomers linked by ester moieties. The consistent and comparable recovery data for losartan and valsartan on Tomtec PDMS 7 in contrast

with Tomtec PDMS 4 and 903 sampling paper shows that the two compounds are not susceptible to matrix effects by the polyester material. This may be because losartan and valsartan belong to the same therapeutic class (angiotensin 2 receptor antagonist) and may therefore have similar physicochemical properties.

LC-HRMS analyses of a blank solution extract of the VAMS device, HemaSpot - HF, Tomtec cotton (PDMS 4), polyester (PDMS 7) cards and 903 card shows similar total ion chromatogram (TIC) profile, apart from the polyester (PDMS 7) card (Figure 7.4). The comparable TIC profile seen for the VAMS device, HemaSpot, tomtec cotton (PDMS 4) and 903 sampling paper is expected because these devices may all be made of cellulose or cotton fibres.

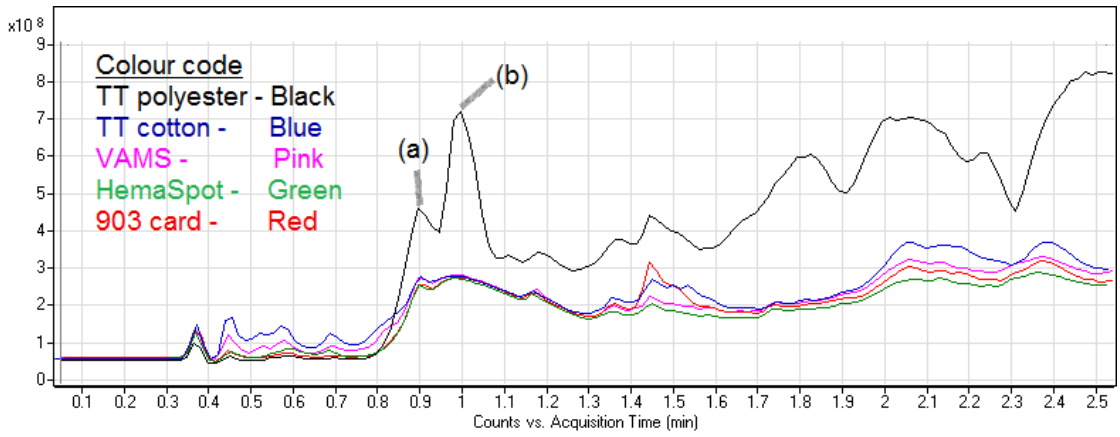


Figure 7.4 An overlaid TIC of a blank device extract of a Tomtec dry media spot slide PDMS 7, PDMS 4, VAMS, HemaSpot and 903 card.

The TIC trace for the polyester (PDMS 7) sampling card is different because the device is made from terephthalic acid and ethylene glycol (Figure 7.5) in a condensation reaction with the loss of water to produce an ester (ethylene terephthalate). This reaction occurs in a repeated fashion since the acid monomer and the alcohol unit each have two functional groups, at the opposite sides of each compound. A simple illustration is demonstrated with [A-B-A-B-A-B] (Figure 7.5). This leads to the formation of the polymerised units of the monomer ethylene terephthalate to a condensed polymer called polyethylene terephthalate (PET) (Figure 7.5).

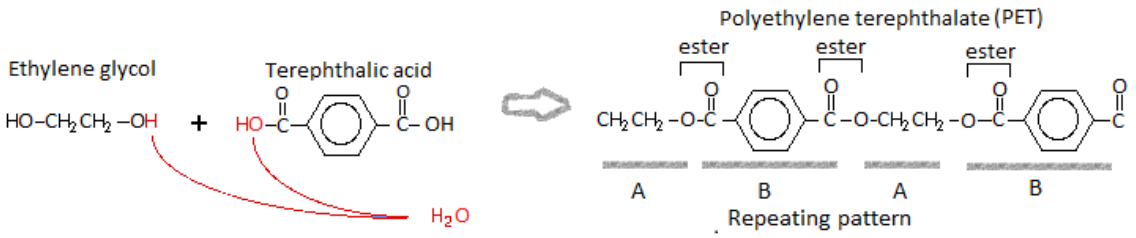


Figure 7.5 Formation of polyethylene terephthalate (PET).

A walk through the chromatogram using the qualitative analysis software version 4.00 (Agilent Technologies), over the peaks labelled (a) at retention time 0.9 minutes and (b) at 1.0 minutes on Figure 7.4 show extracted ion chromatogram (EIC) traces (Figure 7.6 and Figure 7.7) consistent with this polymer. This is because there is a constant addition of a mass of m/z 44.00 representing the ester functional group to the peaks labelled (A) to (I) in Figures 7.6 and 7.7.

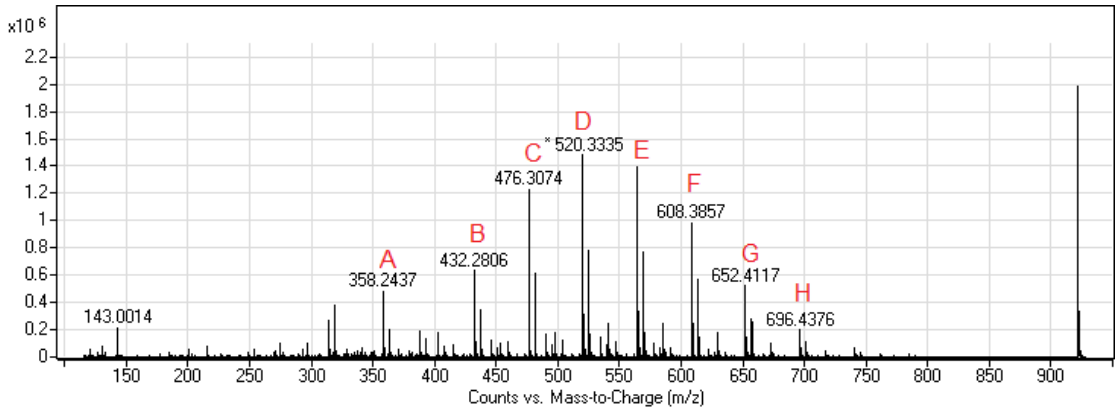


Figure 7.6 Representative EIC of ions appearing from the peak (a) at a retention time of 0.904 minutes on Tomtec PDMS 7.

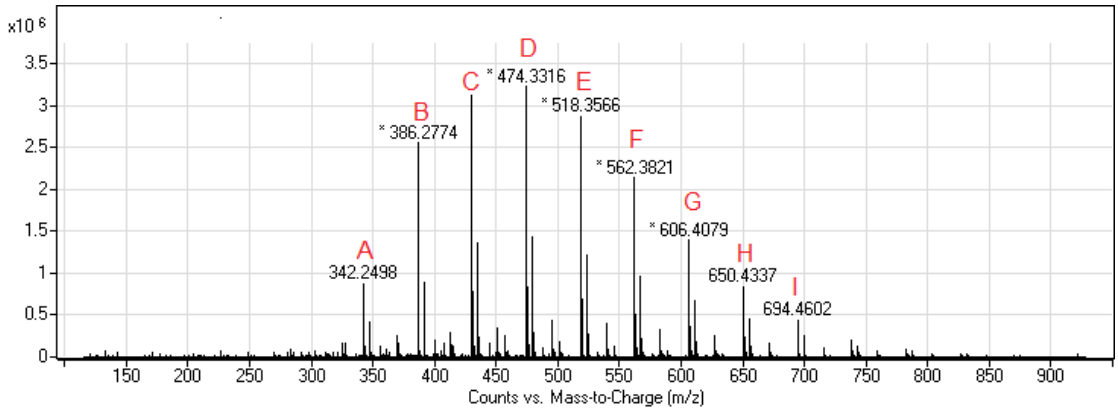


Figure 7.7 Representative EIC of ions appearing from the peak (b) at a retention time of 1.004 minutes on Tomtec PDMS 7.

7.1.3 Ahlstrom 167L cards

The Ahlstrom 167L card is a novel microsampling card reported to eliminate the hematocrit effect. Ahlstrom 167L utilises a punch for analysis and is reported to yield reliable quantitative results irrespective of the blood hematocrit.

In contrast to the conventional 903 sampling paper, qualitative investigations showed that a 30µl blank blood standard produced spots of about ~9.5mm on 903 sampling paper and ~7.5mm on Ahlstrom 167L cards (Figure 7.8a and 7.9a). The difference in spot sizes on the two sampling cards could be attributed to the difference in weight (Table 7.3) and the variability in thickness of the two sampling cards. The mean weight of a blank 8mm disk from 903 and Ahlstrom 167L cards were 9.13mg and 19.19 respectively. In addition, 903 sampling paper has a thickness of ~0.4mm, whilst that of Ahlstrom 167L cards is about ~1.0mm thick. Hence the blood drop spread over a larger surface area on 903 sampling paper, compared to Ahlstrom 167L cards thus producing small size blood spots. Another observation was that, the thickness of the Ahlstrom 167L cards, affected the spread of the blood spot on both sides of the sampling card. Figure 7.8 shows that the size of the blood spots on the (a) front of the card are bigger compared to the size of the blood spots at the (b) back of the same card. However, this effect was not seen with the 903 sampling paper (Figure 7.9), where the spot size was the same on the (a) front and (b) back of the sampling card.

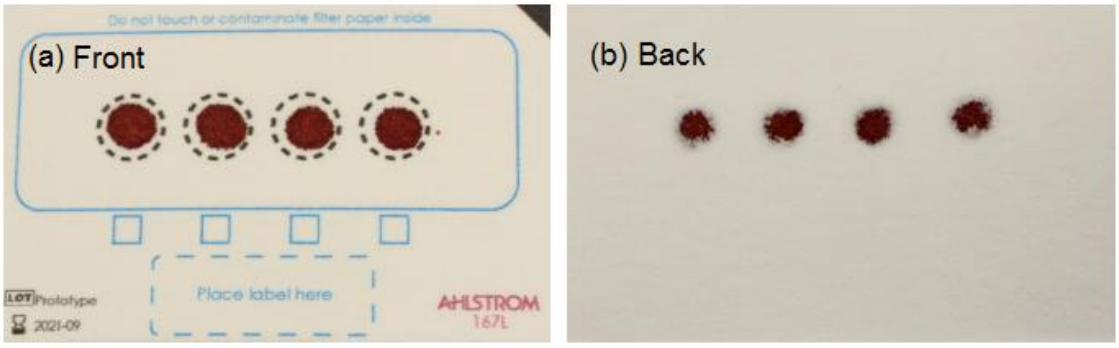


Figure 7.8 30µl spots of blank blood standard on the front (a) and back (b) of Ahlstrom 167L card after 3 hours of drying.

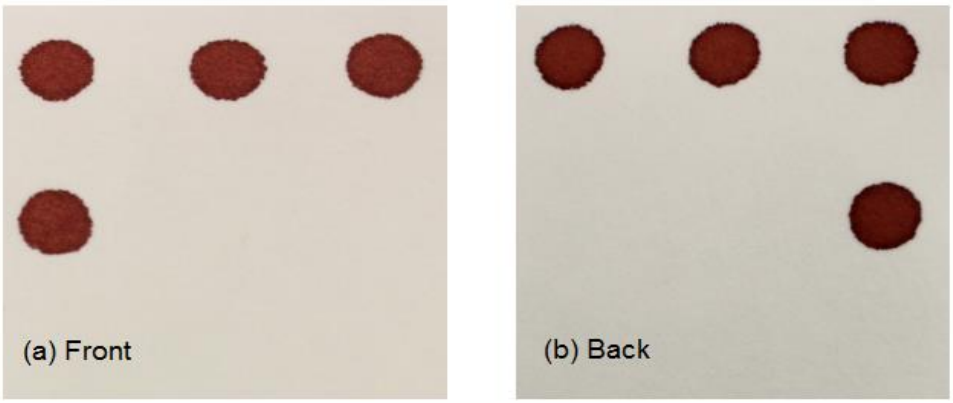


Figure 7.9 30µl spots of blank blood standard on the front (a) and back (b) of 903 sampling card after 3 hours of drying.

Table 7.3 Comparison of an 8mm blank disk of 903 and 167L sampling card.

	Weight of 8mm blank card (mg)	
Disc number	**903 Sampling paper**	**Ahlstrom 167L**
1	9.01	19.63
2	9.22	18.70
3	9.15	19.24
Mean weight	**9.13**	**19.19**

An LC-HRMS analyses of a blank card solution extract from 903 sampling paper and Ahlstrom 167L was performed using the procedure described in Chapter 4, section 4.5.1. A total ion chromatogram (TIC) of the extracted blank 903 card and Ahlstrom 167L card is shown in Figure 7.10.

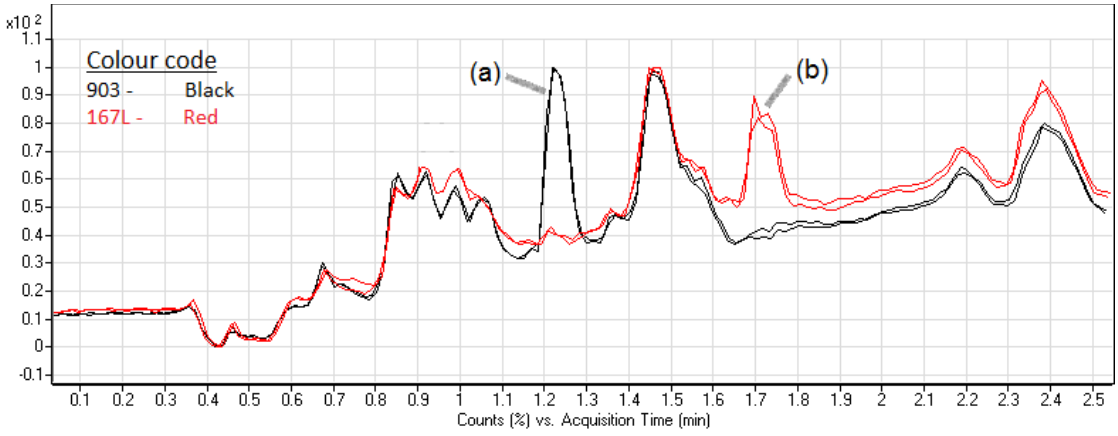


Figure 7.10 Overlaid TIC of a blank 903 paper and Ahlstrom 167L card solution extract.

The overlaid total ion chromatograms reveal obvious differences between the two sampling cards by the peaks marked (a) on 903 sampling paper at a retention time of 1.22 minutes and (b) on Ahlstrom 167L card at a retention time of 1.71 minutes. A walk through the two chromatograms using the qualitative analysis software version 4.00 (Agilent Technologies), was performed to reveal the ions in peaks (a) and (b). An extracted ion chromatogram (EIC) showing the ions in peak (a) and (b) are shown in Figure 7.11 and 7.12 respectively. The EIC of peak (a) on 903 sampling paper shows ions of m/z 148.5350, 219.0478, 318.5819 and 418.1143, which is different from the EIC of peak (b) on Ahlstrom 167L card which shows ions of m/z 147.0809, 371.1210 and 719.2524.

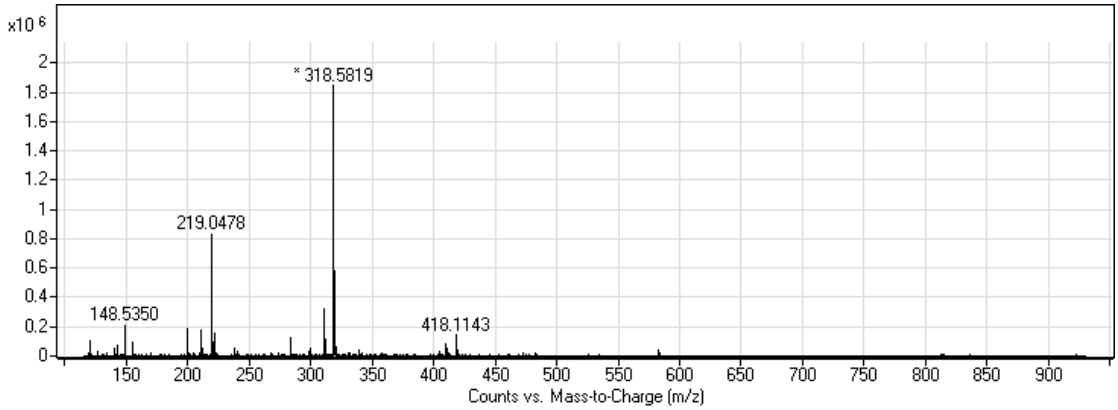


Figure 7.11 Representative EIC of ions appearing from the peak (a) at a retention time of 1.22 minutes on 903 sampling paper.

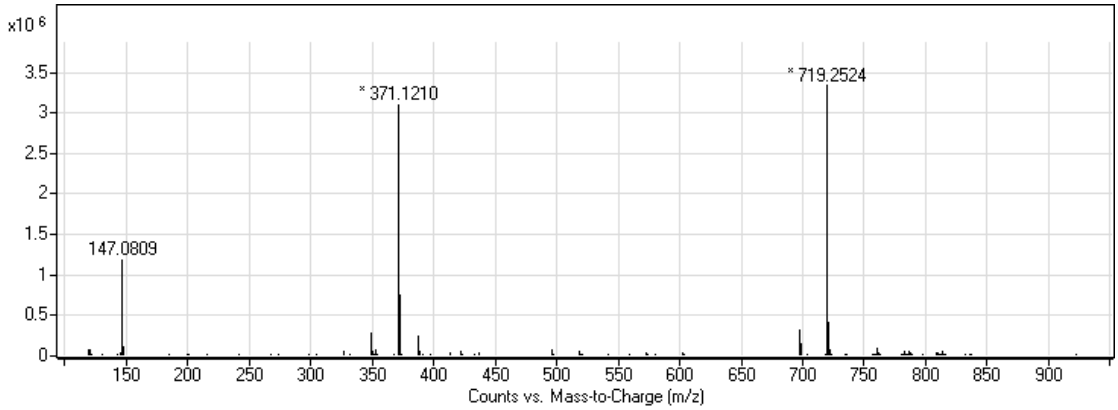


Figure 7.12 Representative EIC of ions appearing from the peak (b) at a retention time of 1.71 minutes on Ahlstrom 167L card.

7.1.4 Volumetric absorptive microsampling

Volumetric absorptive microsampling, as introduced in (Chapter 3, section 3.3.2) is a novel microsampling bead technology developed specifically to collect fixed volumes of blood to eliminate the Hct effect associated with DBS cards. The device consists of a polymeric tip attached to a moulded plastic handle (Chapter 3, Figure 3.2). The process of sample collection involves wicking of a liquid sample onto the porous substrate upon contact. VAMS is used for fixed volume collection of a 10µl or 20µl volume of blood directly from a finger prick eliminating the volumetric haematocrit effect associated with the DBS sampling when a punch is used. VAMS utilise whole sample for analysis and is reported to yield reliable quantitative data (Qu et al., 2017; Kita and Mano, 2017). An example of VAMS is currently marketed as Mitra^{TM} and is available on the market in a clam shell containing either two or four sampling devices (Neoteryx, 2015). The validation and application of VAMS for the collection of volunteer blood samples for the assessment of adherence to CVD prescription medication is presented in Chapters 5 and 6 respectively.

7.1.5. Hemaxis – DB collection device

Hemaxis – DB blood collection device is a new microsampling device that utilises microfluidic technology to collect accurate volumes of 5µl or 10µl of whole blood from a finger or heel prick unto a filter paper. The device is available in two formats depending on spot volume, either a 4 x (5µl) or a 4 x (10µL) version. Hemaxis utilises the whole spot for analysis and thus eliminates the hematocrit bias. The device is made of three main parts (1) a microfluidic chip, (2) a sampling card and (3) a protective case (Figure 7.13). The microfluidic chip with its microsized channels ensures easy blood collection by the user and enables precise volumes to be deposited unto the sampling card housed by the protective case to secure the sample after collection. This makes it easy for blood sampling to be performed by anyone, anywhere and at any time. The sample is then dried at room temperature prior to shipping. The sampling card onto which blood is deposited takes the format of the

conventional 903 Protein Saver card to allow compatibility with automated handling processes in laboratories.

A similar product is currently under development by name (Hemaxis – DP) for the collection of microvolumes of plasma or serum without the use of a centrifuge or filtration membrane (Hemaxis, 2017).

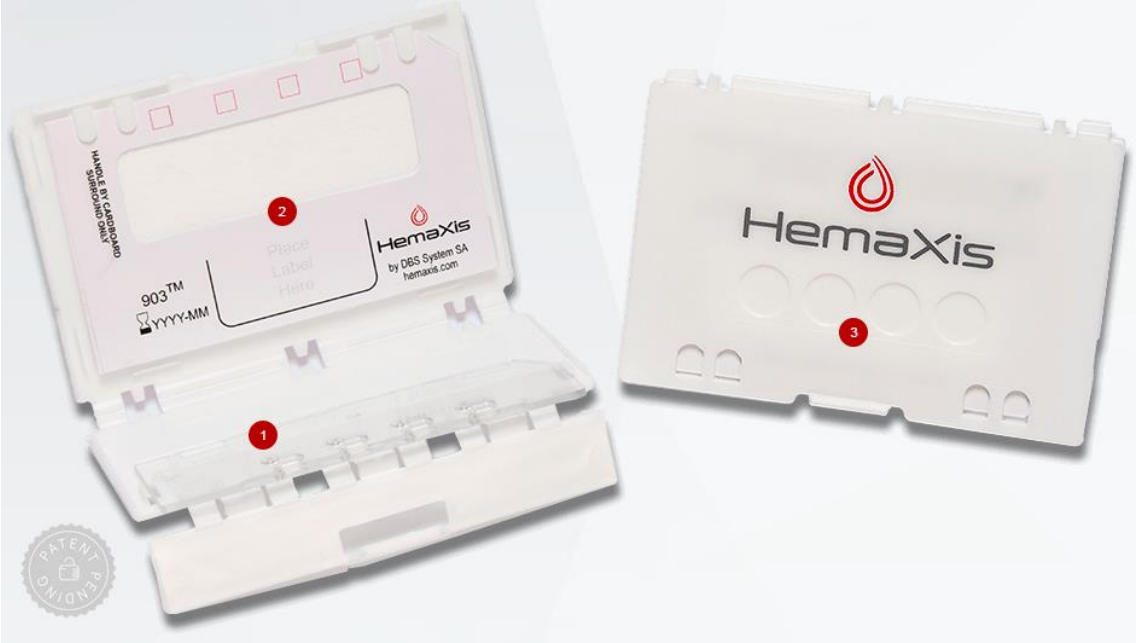


Figure 7.13 A picture of the Hemaxis-DB dried blood collection device (Hemaxis, 2017).

7.1.6 Hemapen

Hemapen is a single use novel microsampling device currently under development. It is designed for precise volume blood collection and storage in DBS format. Sampling involves end-to-end capillary collection of accurate volumes of blood onto pre-cut DBS paper. The DBS paper is securely housed in the integrated compartment of the device to ensure protection of the collected blood sample and prevent contamination between patients. Hemapen is developed to overcome the analytical hurdles associated with conventional DBS sampling such as Hct effect, volumetric inaccuracy and sample integrity. Like all other dried blood microsampling formats, Hemapen enhances blood sampling at remote locations and resource limited areas of the world.

The device has a post-collection locking feature which restricts access to the collected sample to only laboratory staff reducing any biohazard risk to handlers. Some of its unique features include an ergonomic handheld pen design (Figure 7.14) for user convenience and a transparent front end that enables the user to visualise emptying of the capillaries which signifies sample collection completion.

Figure 7.14 Shows a picture of the Hemapen microsampling device (Trajan Scientific & Medical, 2015).

An overlaid total ion chromatogram (TIC) profile for a blank card solution extract from the Hemapen and 903 sampling paper (Figure 7.14), shows that there is no significant differences between the two sampling materials. This is expected because the card material for Hemapen and 903 sampling paper are made of cellulose.

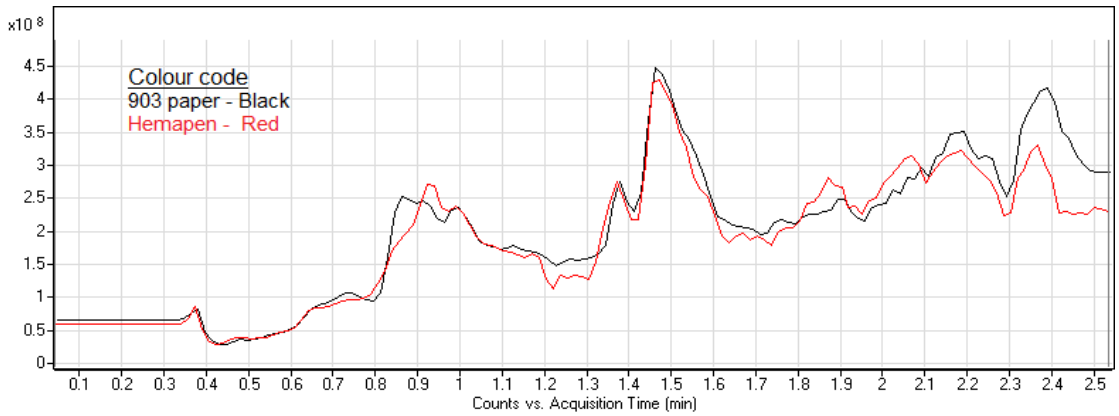


Figure 7.15 Overlaid TIC of a blank 903 paper and Hemapen device solution extract.

7.1.7 Noviplex plasma preparation cards

Noviplex card is a new microsampling tool that has been developed for the rapid generation of fixed volume of plasma from whole blood in about 3 minutes. In contrast with DBS cards, plasma is collected hence there are no hematocrit effects. The device works by collecting a non-volumetric amount of whole blood from a finger or heel prick directly onto an overlay on the noviplex card (Figure 7.16).

The collected blood sample dissipates by capillary action across a spreading layer in the device, where an in-built separator membrane, separates the blood cells from the plasma by means of adsorption and filtration without the need for any capillaries or centrifuging process (Figure 7.16). The generated plasma is then collected on a plasma collection reservoir or removable disk which can be exposed for removal by stripping off the upper layer of the device. The plasma sample is removed after three minutes and dried for about 15 minutes prior to shipment to the laboratory without the need for dry ice.

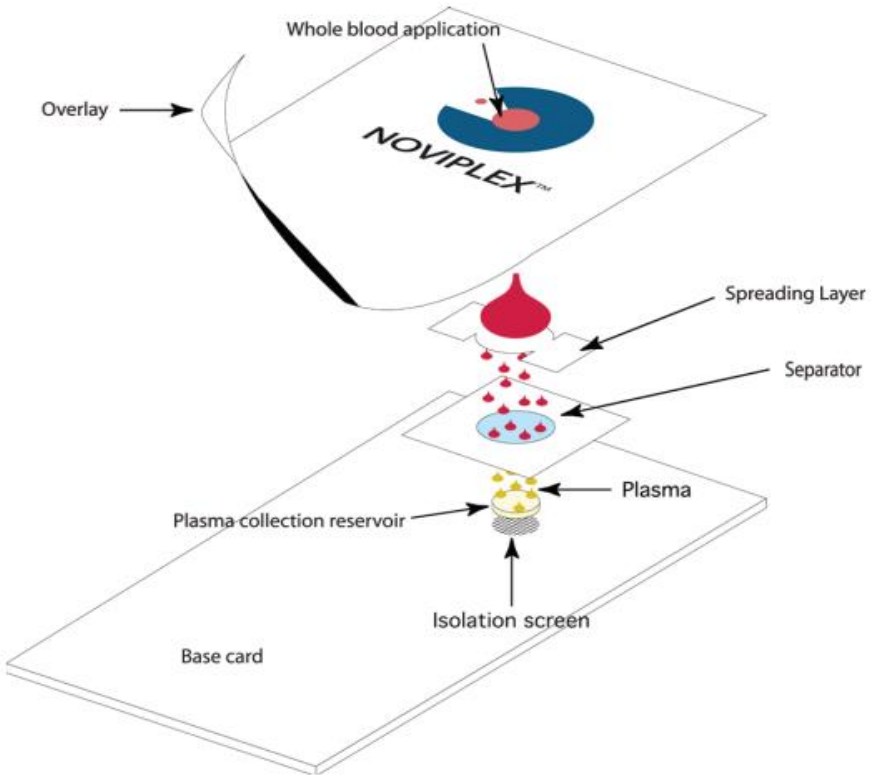


Figure 7.16 A schematic of a Noviplex plasma preparation card (Novilytic limited, 2017).

The device comes in two formats, the Noviplex plasma prep cards and the Noviplex duo plasma prep cards. The former collects a fixed 2.5µl volume of plasma sample from a variable amount of blood (25µl – 75µl), whilst the latter (Noviplex duo card) generates two times (2x) 3.8µl volume of plasma samples from a single application of about (60µl – 100µl) of blood. The Noviplex cards are reported to offer quick sample preparation, whilst maintaining assay reproducibility and selectivity (Novilytic limited, 2017).

7.1.8 Conclusion

Looking at the innovations in microsampling methods discussed in this Chapter, which are all reported to address the hurdles with conventional DBS such as difficulties with self-sampling, volumetric inaccuracies and hematocrit effects, the vision of self-sampling at home, away from a hospital will soon be a reality for various clinical applications including routine therapeutic drug monitoring (TDM). Which is currently limited because of the highly invasive sampling (venepuncture), the need for a phlebotomist and clinical appointments. This will be a huge step not only in objectively assessing patient's adherence to medication, but also in the provision of personalised healthcare. The "self-provided" microvolume sample by the patient may offer healthcare providers with a unique means of providing evidence based and tailor-made pharmacotherapy suited to each patient. Tanna and Lawson (2016) argue that this will help shift the balance of healthcare provision from the hospitals to the community broadening access to healthcare. Thus, application of microsampling methods to TDM and other clinical applications will provide enhanced patient benefits in terms of time, cutting down journeys to phlebotomy appointments, cost of travel and the active involvement of the patient in their own healthcare, as they discuss results from their blood drug level measurements with their healthcare provider. This approached to personalised healthcare could become routine providing huge savings for healthcare providers through better patient outcomes, optimised medication usage, reduction on avoidable hospital readmissions and needless patient deaths.

Chapter 8 Overall conclusions and future work

8.1 Overall conclusion

In this research study, the goal was to develop, validate and apply a microsampling based LC - HRMS assay for multi compound drug determination for the inference of adherence to CVD medication in cardiovascular disease patients. Hence considering the questions raised at the beginning of the research study in Chapter 1, section 1.5, the following conclusions can be drawn:

A. A reliable and cost-effective DBS and VAMS based LC-HRMS assay has been developed and validated for the TDM of eleven candidate cardiovascular drugs in heart disease patients. To date this is the first microanalytical assay developed for the simultaneous determination of multiple (≥10) cardiovascular drugs in volunteer microvolume blood samples and therefore demonstrates novelty.
B. The developed LC-HRMS assay provides information on the levels of medication in the patients' blood. Thus, in the event of poor patient progress to treatment, this information can aid clinicians if for example; the problem is because of poor adherence to treatment, incorrect diagnosis or poor choice of medication.
C. The results from the analyses of volunteer samples have demonstrated that monitoring therapeutic drug levels by direct analyses of patient microvolume blood samples can be a useful tool for dose optimisation and monitoring of drug interactions for individual patients.
D. The research study has also shown that, it is possible to simultaneously quantify a wide panel of commonly used cardiovascular drugs: atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan in microvolume blood samples collected using microsampling methods coupled with LC-HRMS analyses. However, due to the small sample volume and the wide differences in physicochemical properties of

the analytes, challenges with analyte extraction from DBS sample should be addressed accordingly.

E. The study has also demonstrated that application of microsampling methods for the collection of blood samples is a feasible alternative to traditional blood sampling (venepuncture) for the TDM of cardiovascular drugs. It has high patient acceptability in contrast with venepuncture and most importantly offers no restrictions to sample collection because of patient's ability to self-sample. Thus, it is foreseen that the current innovations in microsampling methods will soon make self-sampling for the purposes of routine therapeutic drug monitoring a reality.
F. The study has also demonstrated that indication of adherence to medication based on the levels of CVD drug in the patients' blood is the most reliable marker for cases of nonadherence to CVD drugs since it is evidence based. This is because clinicians may need evidence to quantify the effects of prescribed medication to aid the clinical decision-making process and the data generated from blood drug levels can provide the evidence.
G. The developed microsampling based LC-HRMS assay can be adapted and extended to other medical conditions with known prevalence of nonadherence to medication such as diabetes, depression and cancer.
H. The study also shows that microsampling based LC-HRMS assays has great potential to enable the implementation of routine TDM of cardiovascular medications in everyday clinical practice. Providing an evidence based approach to the assessment of cardiovascular medication adherence.
I. The microsampling based LC-HRMS assay also shows that, high-resolution full scan mass spectrometry analyses offers benefits in the TDM in cases of poor progression. Because all mass spectral data from the analysed sample is collected which makes it possible for the data to be revisited at a later time which may provide additional clinical data.
J. The results have also demonstrated that VAMS overcomes the limitations observed with using conventional DBS cards such as difficulty in self-sampling,

volumetric inaccuracies and the hematocrit effects. For the selected CVD drugs investigated, analyses on VAMS showed no hematocrit effects over a hematocrit range of 35% - 55%, whereas results on DBS cards revealed that hematocrit bias was significant at extreme hematocrit values.

8.2 Future work

This research study has unveiled several opportunities for further work.

A. Using the developed extraction procedure for atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan in microvolume blood samples collected on VAMS and DBS card, it was not possible to extract amlodipine. Thus, a different extraction procedure had to be developed separately for amlodipine. This meant that four dried blood samples were required to analyse samples from volunteers where amlodipine was prescribed with any of the other CVD drugs. Therefore, an extraction method for all the compounds to enable a single run needs to be explored further.
B. Furthermore, since plasma is the gold standard matrix for the quantification of therapeutic drugs, a study to determine the ratio between DBS and plasma derived concentrations for the selected cardiovascular drugs should have been performed. Hence a bridging study to determine the ratio between (DBS and VAMS) and plasma derived concentration for the selected drugs should be considered in the future so that comparison between matrices is possible. This will further confirm that microsampling methods maintain the integrity of the original sample and represents an accurate surrogate of the original sample.
C. It was also not feasible to validate the developed assay for the selected cardiovascular drugs on all the new microsampling methods discussed in Chapter 7. Thus, further work to validate the assay on HemaSpot-HF device, Ahlstrom 167L cards, Hemaxis – DB, Tomtec PDMS 4 and the HemaPen sample collection device should be considered in the future to allow comparison of data using these microsampling methods.

D. The developed microsampling based LC-HRMS assay, has now gone outside the boundaries of the UK and is currently under investigation for implementation in clinical practice in Iraq. However, for the developed microsampling based LC-HRMS assay to be successfully implemented in clinical practice, on-line extraction which involves the automation of sample preparation and the extraction process must be explored for faster analysis. Currently an offline sample extraction procedure is employed which involves manual punching of disk from the dried blood sample on 903 cards followed by addition of solvents to punched disk, vortexing, centrifuging, evaporation of supernatant and reconstitution of dried residue in solvent for analysis. This process may take a longer time if hundreds of patient samples must be analysed at the clinic. Hence the time taken to prepare the microvolume dried blood sample for analysis must be significantly reduced to enhance sample processing time and increase analytical throughput.

E. The analysis of hundreds of patient samples will lead to the generation of big data which will slow down data processing due to current limitations with software and data storage. Hence MS instruments need to be embedded with the capability for data processing power to generate the data for analyses within the shortest possible time. Since a long waiting period for results could mean patient may be dead before the results is out. Thus, a move towards cloud storage is foreseeable and should be explored by instrument manufacturers.

F. The increase in life expectancy has led to an ageing elderly population. This presents with huge challenges for healthcare systems because the accumulation of chronic diseases rises with older ages. Cardiovascular disease for example affects mainly the elderly. This results in high rates of multimorbidity where cardiovascular disease patients suffer from other conditions such as diabetes and depression. Multimorbidity leads to polypharmacy prescribing where patients are prescribed not less than 5 different medicines at a time. This shoots up the economic burden on health

systems since the government must foot the bill for the medications prescribed for the elderly population. Hence medicine optimisation becomes critical to ensure the cost effectiveness of treatment in the elderly population for agencies like the National Institute for Health and Care Excellence (NICE) that set guidelines for the NHS to regulate cost of treatment. Thus, for efficient service delivery in clinical practice, expansion of the developed microsampling based LC-HRMS assay to screen for other medications that cardiovascular disease patients may be taking, for example oral antidiabetic and antidepressant drugs will help with the implementation of precision medicine. This will lead to patient care improvement and a reduction in medication wastage in healthcare provision and must be explored in the future.

G. Furthermore, though the mass spectrometer has undergone massive transformation over the last three decades to reduce its size, it is still a huge bench top instrument which will not fit into every ward at the clinic. Hence manufacturers will need to reduce the size of the instrument into a portable or mobile bedside piece of equipment which is affordable and can easily be installed into hospital wards and clinics. In addition, transformation of the mass spectrometer into an integrated user-friendly push button unit that can take a biological sample, prepare the sample, perform analyses and produce reports will make service delivery efficient in clinical practice. Hence more efforts are required from manufacturers to make this possible.

References

Abdulazeez, F.I., Omole, M. and Ojulari, S.L., 2014. Medication adherence amongst diabetic patients in a tertiary healthcare institution in central Nigeria. *Tropical Journal of Pharmaceutical Research*, *13*(6), pp.997-1001.

Abernethy, D.R., 1992. Pharmacokinetics and pharmacodynamics of amlodipine. *Cardiology*, *80*(1), pp.31-36.

Abolbashari, M., Macaulay, T.E., Whayne, T.F., Mukherjee, D. and Saha, S., 2017. Polypharmacy in Cardiovascular Medicine: Problems and Promises. *Cardiovascular & Hematological Agents in Medicinal Chemistry*. 15(1), pp.31-39.

AbuRuz, S., Millership, J. and McElnay, J., 2006. Dried blood spot liquid chromatography assay for therapeutic drug monitoring of metformin. *Journal of Chromatography B*, *832*(2), pp.202-207.

Adler, A.J., Martin, N., Mariani, J., Tajer, C.D., Owolabi, O.O., Free, C., Serrano, N.C., Casas, J.P. and Perel, P., 2017. Mobile phone text messaging to improve medication adherence in secondary prevention of cardiovascular disease. *The Cochrane Library*, pp.3 – 59.

Ahmad, M., Qamar-Uz-Zaman, M., Madni, A., Usman, M., Atif, M., Akhtar, N. and Murtaza, G., 2011. Pharmacokinetic and Bioavailability Studies of Commercially Available Simvastatin Tablets in Healthy and Moderately Hyperlipidemic Human Subjects. *Journal of the Chemical Society of Pakistan, 33*(1), pp. 49-54.

Ahmed, R. and Aslani, P., 2014. What is patient adherence? A terminology overview. *International Journal of Clinical Pharmacy*, *36*(1), pp.4-7.

Ahmed, T.A., Horn, J., Hayslip, J. and Leggas, M., 2012. Validated LC–MS/MS method for simultaneous determination of Simvastatin and its acid form in human plasma and cell lysate: Pharmacokinetic application. *Journal of Pharmaceutical Analysis*, *2*(6), pp.403-411.

Alakhali, K.M., 2013. Method Validation for Analysis of Simvastatin in Human Plasma Using Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS). *Journal of Clinical and Diagnostic Research: JCDR*, *7*(12), pp.2739-2743.

Alcaide, M.L., Ramlagan, S., Rodriguez, V.J., Cook, R., Peltzer, K., Weiss, S.M., Sifunda, S. and Jones, D.L., 2017. Self-Report and Dry Blood Spot Measurement of Antiretroviral Medications as Markers of Adherence in Pregnant Women in Rural South Africa. *AIDS and Behavior*, *21*(7), pp.2135-2140.

Al-Ghazawi, M. and AbuRuz, S., 2010. Determination of ciprofloxacin in dried blood spots for therapeutic drug monitoring. *Chromatographia*, *71*(11-12), pp.999-1005.

Alkhateeb, F., 2015. *Novel Strategies for Rapid Analytical Preparation of Solid Samples* (Doctoral thesis, University of Calgary).pp.3-35.

Allanson, A.L., Cotton, M.M., Tettey, J.N.A. and Boyter, A.C., 2007. Determination of rifampicin in human plasma and blood spots by high performance liquid chromatography with UV detection: a potential method for therapeutic drug monitoring. *Journal of Pharmaceutical and Biomedical Analysis*, *44*(4), pp.963-969.

Alsarra, I.A., 2009. High-performance liquid chromatographic method for quantitative determination of amlodipine in human plasma and pharmaceutical dosage form and its application to pharmacokinetic studies. *Journal of Chromatographic Science, 47*, pp.863-867.

Anderson, J.R. and Nawarskas, J.J., 2001. Cardiovascular drug-drug interactions. *Cardiology Clinics*, *19*(2), pp.215-234.

Androw, P.L., 1997. Atorvastatin A review of its pharmacology and therapeutic potential in the management of hyperlipidemias. *Drug; 53,* pp.828 - 847.

Anon British Heart Foundation Statistics Database UK, 2013. Economic cost. Coronary Heart Disease, economic cost. https://www.bhf.org.uk/, (accessed 13.07.16).

Aonuma, K., Shiga, T., Atarashi, H., Doki, K., Echizen, H., Hagiwara, N., Hasegawa, J., Hayashi, H., Hirao, K., Ichida, F. and Ikeda, T., 2017. Guidelines for Therapeutic Drug

Monitoring of Cardiovascular Drugs Clinical Use of Blood Drug Concentration Monitoring (JCS 2015) —Digest Version—. *Circulation Journal*, *81*(4), pp.581-612.

Apostolou, C., Kousoulos, C., Dotsikas, Y., Soumelas, G.S., Kolocouri, F., Ziaka, A. and Loukas, Y.L., 2008. An improved and fully validated LC–MS/MS method for the simultaneous quantification of simvastatin and simvastatin acid in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, *46*(4), pp.771-779.

Araujo, P., 2009. Key aspects of analytical method validation and linearity evaluation. *Journal of Chromatography B*, *877*(23), pp.2224-2234.

Aslani, P. and Schneider, M.P., 2014. Adherence: the journey of medication taking, are we there yet? *International Journal of Clinical Pharmacy, 36*(1), pp.1-3.

Awad, A., Osman, N. and Altayib, S., 2017. Medication adherence among cardiac patients in Khartoum State, Sudan: a cross-sectional study. *Cardiovascular Journal of Africa*, *28*, pp.001-007.

Baggarly, S.A., Kemp, R.J., Wang, X. and Magoun, A.D., 2014. Factors associated with medication adherence and persistence of treatment for hypertension in a Medicaid population. *Research in Social and Administrative Pharmacy*, *10*(6), pp.e99-e112.

Bain, K.T., 2010. Public health implications of household pharmaceutical waste in the United States. *Health Services Insights*, *3*, pp.21.

Barco, S., Castagnola, E., Moscatelli, A., Rudge, J., Tripodi, G. and Cangemi, G., 2017. Volumetric adsorptive microsampling-liquid chromatography tandem mass spectrometry assay for the simultaneous quantification of four antibiotics in human blood: Method development, validation and comparison with dried blood spot. *Journal of Pharmaceutical and Biomedical Analysis*, *145*, pp.704-710.

Barnett, N.L., 2014. Medication adherence: where are we now? A UK perspective. *European Journal of Hospital Pharmacy: Science and Practice*, *21*(3), pp.181-184.

Baroletti, S. and Dell'Orfano, H., 2010. Medication adherence in cardiovascular disease. *Circulation*, *121*(12), pp.1455-1458.

Barrett, B., Huclova, J., Bořek-Dohalský, V., Němec, B. and Jelinek, I., 2006. Validated HPLC–MS/MS method for simultaneous determination of simvastatin and simvastatin hydroxy acid in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, *41*(2), pp.517-526.

Bateman, K.P., Kellmann, M., Muenster, H., Papp, R. and Taylor, L., 2009. Quantitative–qualitative data acquisition using a benchtop Orbitrap mass spectrometer. *Journal of the American Society for Mass Spectrometry*, *20*(8), pp.1441-1450.

Bathula, S.N.P. and Devani, K.P., 2011. Quantitative Determination of Amlodipine in Human Plasma by Ultra Performance Liquid Chromatography-Electro Spray Ionisation Mass Spectrometry: Application to a Clinical Pharmacokinetic Study. *Asian Journal of Pharmaceutical and Clinical Research*, *5*(1), pp.89-93.

Baviskar, D., Sharma, R. and Jain, D., 2009. High-Performance Liquid Chromatography Method for Determination of Diltiazem in Human Plasma. *Asian Journal of Chemistry*, *21*(7), pp.5048-5054.

Beermann, B., 1988. Pharmacokinetics of lisinopril. *The American Journal of Medicine*, *85*(3), pp.25-30.

Bellosta, S., Paoletti, R. and Corsini, A., 2004. Safety of statins. *Circulation*, *109*(1), pp.111-150.

Bendtsen, F. and Henriksen, J.H., 1989. Comparative pharmacokinetics and pharmacodynamics of lisinopril and enalapril, alone and in combination with propranolol. *Journal of Human Hypertension*, *3*(1), pp.139-145.

Bernieh, D., Lawson, G. and Tanna, S., 2017. Quantitative LC–HRMS determination of selected cardiovascular drugs, in dried blood spots, as an indicator of adherence to medication. *Journal of Pharmaceutical and Biomedical Analysis*, *142*, pp.232-243.

Bews, H.J., Carlson, J.C., Jha, A., Basu, S., Halayko, A.J. and Wong, C.S., 2014. Simultaneous quantification of simvastatin and simvastatin hydroxy acid in blood serum at physiological pH by ultrahigh performance liquid chromatography–tandem mass spectrometry (UHPLC/MS/MS). *Journal of Chromatography B*, *947*, pp.145-150.

Bhatt, J., Singh, S., Subbaiah, G., Shah, B., Kambli, S. and Ameta, S., 2007. A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the estimation of amlodipine in human plasma. *Biomedical Chromatography*, *21*(2), pp.169-175.

Bhatt, J., Subbaiah, G., Kambli, S., Shah, B., Patel, M., Saxena, A., Baliga, A., Nigam, S., Parekh, H. and Yadav, G., 2007. A high throughput and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the estimation of bisoprolol in human plasma using multiplexing technique. *Journal of Chromatography B*, *852*(1), pp.374-381.

Bindschedler, M., Degen, P., Flesch, G., De Gasparo, M. and Preiswerk, G., 1997. Pharmacokinetic and pharmacodynamic interaction of single oral doses of valsartan and furosemide. *European Journal of Clinical Pharmacology*, *52*(5), pp.371-378.

Bitton, A., Choudhry, N.K., Matlin, O.S., Swanton, K. and Shrank, W.H., 2013. The impact of medication adherence on coronary artery disease costs and outcomes: a systematic review. *The American Journal of Medicine*, *126*(4):357, pp.e7-e27.

Blessborn, D., Romsing, S., Bergqvist, Y. and Lindegardh, N., 2010. Assay for screening for six antimalarial drugs and one metabolite using dried blood spot sampling, sequential extraction and ion-trap detection. *Bioanalysis*, *2*(11), pp.1839-1847.

Boehme, P., Wienand, P., Herrmann, M., Truebel, H. and Mondritzki, T., 2017. New digital adherence devices could prevent millions of strokes from atrial fibrillation by the end of the next century. *Medical Hypotheses*, *108*, pp.46-50.

Boillot, F., Serrano, L., Muwonga, J., Kabuayi, J.P., Kambale, A., Mutaka, F., Fujiwara, P.I., Decosas, J., Peeters, M. and Delaporte, E., 2016. Programmatic feasibility of dried blood spots for the virological follow-up of patients on antiretroviral treatment in Nord

Kivu, Democratic Republic of the Congo. *Journal of Acquired Immune Deficiency Syndrome*, *71*(1), pp.e9-e15.

Boswell, K.A., Cook, C.L., Burch, S.P., Eaddy, M.T. and Cantrell, C.R., 2012. Associating medication adherence with improved outcomes: a systematic literature review. *American Journal of Pharmacy Benefits*, *4*(4), pp.e97-e108.

Bosworth, H.B., Granger, B.B., Mendys, P., Brindis, R., Burkholder, R., Czajkowski, S.M., Daniel, J.G., Ekman, I., Ho, M., Johnson, M. and Kimmel, S.E., 2011. Medication adherence: a call for action. *American Heart Journal*, *162*(3), pp.412-424.

Bowen, C.L., Kehler, J., Boram, S., Abberley, L., Szapacs, M. and Evans, C., 2016. Modify on the fly: triple quad to high resolution in support of a dermal clinical study requiring an ultra low LLOQ. *Bioanalysis*, *8*(3), pp.205-214.

Bowen, C.L., Licea-Perez, H., Karlinsey, M.Z., Jurusik, K., Pierre, E., Siple, J., Kenney, J., Stokes, A., Spooner, N. and Evans, C.A., 2013. A novel approach to capillary plasma microsampling for quantitative bioanalysis. *Bioanalysis*, *5*(9), pp.1131-1135.

Boyd, R.A., Chin, S.K., Don-Pedro, O., Verotta, D., Sheiner, L.B., Williams, R.L. and Giacomini, K.M., 1989. The pharmacokinetics and pharmacodynamics of diltiazem and its metabolites in healthy adults after a single oral dose. *Clinical Pharmacology and Therapeutics*, *46*(4), pp.408-419.

Brandstetter, S., Finger, T., Fischer, W., Brandl, M., Böhmer, M., Pfeifer, M. and Apfelbacher, C., 2017. Differences in medication adherence are associated with beliefs about medicines in asthma and COPD. *Clinical and Translational Allergy*, *7*(1), pp.39-46.

Breidinger, S. and Woolf, E., 2017. Regulated Bioanalysis—Historical Aspects and Key Concepts Related to Its Use. In *Regulated Bioanalysis: Fundamentals and Practice,* Springer International Publishing, pp.87-102.

Brinker, S., Pandey, A., Ayers, C., Price, A., Raheja, P., Arbique, D., Das, S.R., Halm, E.A., Kaplan, N.M. and Vongpatanasin, W., 2014. Therapeutic drug monitoring facilitates

blood pressure control in resistant hypertension. *Journal of the American College of Cardiology*, *63*(8), pp.834-835.

British Broadcasting Corporation (BBC), News article, patients face 'distressing readmissions', 2017 http://www.bbc.co.uk/news/health-41740525, (assessed 30.10.17).

British Heart Foundation UK, Cardiovascular Disease Statistics, 2015. https://www.bhf.org.uk/publications/statistics/cvd-stats-2015/, (assessed 13.07.16).

British National Formulary (BNF) 2016, cardiovascular drugs, https://bnf.nice.org.uk/treatment-summary/antihypertensive-drugs.html, (assessed 04.10.14).

Brown, M.T. and Bussell, J.K., 2011, April. Medication adherence: WHO cares? In *Mayo Clinic Proceedings, Elsevier*, *86*(4), pp. 304-314.

Bruxvoort, K., Goodman, C., Kachur, S.P. and Schellenberg, D., 2014. How patients take malaria treatment: a systematic review of the literature on adherence to antimalarial drugs. *PloS One*, *9*(1), pp.e84555 – e84570.

Burugula, L., Mullangi, R., Pilli, N.R., Makula, A., Lodagala, D.S. and Kandhagatla, R., 2013. Simultaneous determination of sitagliptin and simvastatin in human plasma by LC-MS/MS and its application to a human pharmacokinetic study. *Biomedical Chromatography*, *27*(1), pp.80-87.

Bylda, C., Thiele, R., Kobold, U. and Volmer, D.A., 2014. Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS. *Analyst*, *139*(10), pp.2265-2276.

Cambien, F., 1994. The angiotensin-converting enzyme (ACE) genetic polymorphism: its relationship with plasma ACE level and myocardial infarction. *Clinical Genetics*, *46*(1), pp.94-101.

Cape, S.S., Meng, M., Koupaei-Abyazani, M.R. and Fast, D.M., 2017. Liquid Chromatography–Mass Spectrometry Assays in the Regulated Bioanalytical Laboratory. In *Regulated Bioanalysis: Fundamentals and Practice*. Springer International Publishing, pp. 121-153.

Carlucci, G., Mazzeo, P., Biordi, L. and Bologna, M., 1992. Simultaneous determination of simvastatin and its hydroxy acid form in human plasma by high-performance liquid chromatography with UV detection. *Journal of Pharmaceutical and Biomedical Analysis*, *10*(9), pp.693-697.

Castillo-Mancilla, J.R., Zheng, J.H., Rower, J.E., Meditz, A., Gardner, E.M., Predhomme, J., Fernandez, C., Langness, J., Kiser, J.J., Bushman, L.R. and Anderson, P.L., 2013. Tenofovir, emtricitabine, and tenofovir diphosphate in dried blood spots for determining recent and cumulative drug exposure. *AIDS Research and Human Retroviruses*, *29*(2), pp.384-390.

CDC. Laboratory Quality Assurance and Standardization Programs, 1993. Guidelines for the collection and shipment of Dried Blood Spot Specimens. USA: Centers for Disease Control and Prevention. https://www.cdc.gov/labstandards/pdf/nsqap/Bloodspot_Transportation_Guidelines.pdf, (accessed 10.09.2017).

Chai, P.R., Castillo-Mancilla, J., Buffkin, E., Darling, C., Rosen, R.K., Horvath, K.J., Boudreaux, E.D., Robbins, G.K., Hibberd, P.L. and Boyer, E.W., 2015. Utilizing an ingestible biosensor to assess real-time medication adherence. *Journal of Medical Toxicology*, *11*(4), pp.439-444.

Chan-Mei, L., Wei, C.M., Bu, F.L., Chen, R., Wang, X.L., Li, R., Wang, B.J. and Guo, R.C., 2013. Determination of amlodipine in human plasma by LC-MS/MS and its bioequivalence study in healthy Chinese subjects. *Pharmacology & Pharmacy*, *4*(02), pp.191-200.

Chemicalize, 2017, https://chemicalize.com, (assessed 06.11.17).

Chen, G., Jirjees Sr, F., Al Bawab, A. and McElnay, J.C., 2018. Quantification of amlodipine in dried blood spot samples by high performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography B*, *1072*, pp.252-258.

Chen, S.L., Lee, W.L., Liang, T. and Liao, I.C., 2014. Factors associated with gender differences in medication adherence: a longitudinal study. *Journal of Advanced Nursing*, *70*(9), pp.2031-2040.

Chepyala, D., Tsai, I.L., Liao, H.W., Chen, G.Y., Chao, H.C. and Kuo, C.H., 2017. Sensitive screening of abused drugs in dried blood samples using ultra-high-performance liquid chromatography-ion booster-quadrupole time-of-flight mass spectrometry. *Journal of Chromatography A*, *1491*, pp.57-66.

Chisholm-Burns, M.A. and Spivey, C.A., 2012. The cost of medication nonadherence: consequences we cannot afford to accept. *Journal of the American Pharmacists Association*, *52*(6), pp.823-826.

Choi, Y., Kim, J.K., Ban, E., Park, J.S. and Kim, C.K., 2008. Determination of losartan in human plasma by liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS): application to bioequivalence study. *Journal of Liquid Chromatography and Related Technologies*, *31*(17), pp.2643-2656.

Chun, I., Yoon, S., Gwak, H. and Kwon, Y., 2006. Pharmacokinetics of doxazosin tablet after multiple-dose administration. *Drug Metabolism Reviews,* 38, pp.152-153.

Chung, M., Vashi, V., Puente, J., Sweeney, M. and Meredith, P., 1999. Clinical pharmacokinetics of doxazosin in a controlled-release gastrointestinal therapeutic system (GITS) formulation. *British Journal of Clinical Pharmacology*, *48*(5), p.678-687.

City Hospital Birmingham, vitamin D testing service, http://www.vitamindtest.org.uk, (assessed 30.05.17).

Clifford, S. and Coyne, K.S., 2014. What is the value of medication adherence? Journal of Managed Care Pharmacy, *20*(7), pp.650-651.

Coleman, D., Smith, G., Lawrence, R., McManus, D., Diaram, S. and Edwards, J., 2017. Capillary microsampling in nonclinical safety assessment: practical sampling and bioanalysis from a CRO perspective. *Bioanalysis*, 9(10), pp787-798.

Collier, C., Jones, E., Pugh-Jones, J., Haines, K., Bracchi, R., Deslandes, P. and Routledge, P., 2017. Polypharmacy: Guidance for prescribing. *British Journal of General Practice,* pp.1-7.

Conway, E.L., McNeil, J.J., Hurley, J., Jackman, G.P., Krum, H., Howes, L.G. and Louis, W.J., 1993. The effects of food on the oral bioavailability of doxazosin in hypertensive subjects. *Drug Investigation*, *6*(2), pp.90-95.

D'Amato, S., 2008. Improving patient adherence with oral chemotherapy. *Oncology Issues*, *23*(4), pp.42-45.

Damon, D.E., Davis, K.M., Moreira, C.R., Capone, P., Cruttenden, R. and Badu-Tawiah, A.K., 2016. Direct biofluid analysis using hydrophobic paper spray mass spectrometry. *Analytical chemistry*, *88*(3), pp.1878-1884.

Danafar, H. and Hamidi, M., 2016. Method validation of amlodipine and atorvastatin by liquid chromatography–mass spectrometry (LC–MS) method in human plasma. *Cogent Medicine*, *3*(1), pp.1129790.

Das, S., Fleming, D.H., Mathew, B.S., Winston A, B., Prabhakar, A.T. and Alexander, M., 2017. Determination of serum carbamazepine concentration using dried blood spot specimens for resource-limited settings. *Hospital Practice*, *45*(2), pp.46-50.

Dasandi, B. and Shah, S., 2009. Development and validation of a high throughput and robust LC–MS/MS with electrospray ionization method for simultaneous quantitation of diltiazem and its two metabolites in human plasma: Application to a bioequivalence study. *Journal of Chromatography B*, *877*(8-9), pp.791-798.

De Abreu, L.R.P., de Castro, S.A.C. and Pedrazzoli, J., 2003. Atenolol quantification in human plasma by high-performance liquid chromatography: Application to bioequivalence study. *The AAPS Journal*, *5*(2), pp.116-122.

De Kesel, P.M., Lambert, W.E. and Stove, C.P., 2015. Does volumetric absorptive microsampling eliminate the hematocrit bias for caffeine and paraxanthine in dried blood samples? A comparative study. *Analytica Chimica Acta*, *881*, pp.65-73.

De Nicolò, A., Avataneo, V., Rabbia, F., Bonifacio, G., Cusato, J., Tomasello, C., Perlo, E., Mulatero, P., Veglio, F., Di Perri, G. and D'Avolio, A., 2016. UHPLC–MS/MS method with protein precipitation extraction for the simultaneous quantification of ten antihypertensive drugs in human plasma from resistant hypertensive patients. *Journal of Pharmaceutical and Biomedical Analysis*, *129*, pp.535-541.

De Nicolò, A., Avataneo, V., Rabbia, F., Sciandra, M., Tosello, F., Cusato, J., Perlo, E., Mulatero, P., Veglio, F., Di Perri, G. and D'avolio, A., 2017. UHPLC–MS/MS method with sample dilution to test therapeutic adherence through quantification of ten antihypertensive drugs in urine samples. *Journal of Pharmaceutical and Biomedical Analysis*, *142*, pp.279-285.

De Vries, R., Barfield, M., Van de Merbel, N., Schmid, B., Siethoff, C., Ortiz, J., Verheij, E., Van Baar, B., Cobb, Z., White, S. and Timmerman, P., 2013. The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium. *Bioanalysis*, *5*(17), pp.2147-2160.

Deep, A., Kumar, P., Kumar, A. and Thakkar, A., 2012. Dry blood spot technique: A review. *International Journal of Pharmaceutical Science Review and Research, 15*(2), pp.90-94.

Delamater, A.M., 2006. Improving patient adherence. *Clinical Diabetes*, *24*(2), pp.71-77.

Denniff, P. and Spooner, N., 2010. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. *Bioanalysis. 2*(8), pp.1385-1395.

Denniff, P. and Spooner, N., 2014. Volumetric absorptive microsampling: a dried sample collection technique for quantitative bioanalysis. *Analytical chemistry*, *86*(16), pp.8489-8495.

Dhaliwal, K.K., King-Shier, K., Manns, B.J., Hemmelgarn, B.R., Stone, J.A. and Campbell, D.J., 2017. Exploring the impact of financial barriers on secondary prevention of heart disease. *BMC Cardiovascular Disorders*, *17*(1), pp.61-69.

Di Bari, M. and Balzi, D., 2017. Cardiovascular Disease in the Oldest Old: A Geriatric Epidemiology Perspective. In *Cardiac Management in the Frail Elderly Patient and the Oldest Old*, Springer International Publishing. pp.9-22.

Dias, E., Hachey, B., McNaughton, C., Nian, H., Yu, C., Straka, B., Brown, N.J. and Caprioli, R.M., 2013. An LC–MS assay for the screening of cardiovascular medications in human samples. *Journal of Chromatography B*, *937*, pp.44-53.

Dillen, L., Cools, W., Vereyken, L., Lorreyne, W., Huybrechts, T., de Vries, R., Ghobarah, H. and Cuyckens, F., 2012. Comparison of triple quadrupole and high-resolution TOF-MS for quantification of peptides. *Bioanalysis*, (5), pp.565-579.

DiMatteo, M.R., 2004. Variations in patients' adherence to medical recommendations: a quantitative review of 50 years of research. *Medical Care*, *42*(3), pp.200-209.

Ding, L., Zhou, X., Guo, X., Song, Q., He, J. and Xu, G., 2007. LC–ESI-MS method for the determination of bisoprolol in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, *44*(2), pp.520-525.

Draft guidance for industry: bioanalytical method validation, US Department of Health and Human Services, Food and Drugs Administration, 2013. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf/, (accessed 15.07.16).

Drayer, D.E., 1986. Pharmacodynamic and pharmacokinetic differences between drug enantiomers in humans: an overview. *Clinical Pharmacology and Therapeutics*, *40*(2), pp.125-133.

Du, L., Cheng, Z., Zhang, Y., Li, Y. and Mei, D., 2017. The impact of medication adherence on clinical outcomes of coronary artery disease: A meta-analysis. *European Journal of Preventive Cardiology*, *24*(9), pp.962-970.

Echizen, H. and Eichelbaum, M., 1986. Clinical pharmacokinetics of verapamil, nifedipine and diltiazem. *Clinical Pharmacokinetics*, *11*(6), pp.425-449.

Elliott, H.L., Meredith, P.A. and Reid, J.L., 1987. Pharmacokinetic overview of doxazosin. *The American Journal of Cardiology*, *59*(14), pp.G78-G81.

Enderle, Y., Foerster, K. and Burhenne, J., 2016. Clinical feasibility of dried blood spots: Analytics, validation, and applications. *Journal of Pharmaceutical and Biomedical Analysis*, *130*, pp.231-243.

Fan, L. and Lee, J.A., 2012. Managing the effect of hematocrit on DBS analysis in a regulated environment. *Bioanalysis*, *4*(4), pp.345-347.

Ferdinand, K.C., Senatore, F.F., Clayton-Jeter, H., Cryer, D.R., Lewin, J.C., Nasser, S.A., Fiuzat, M. and Califf, R.M., 2017. Improving medication adherence in cardiometabolic disease: practical and regulatory implications. *Journal of the American College of Cardiology*, *69*(4), pp.437-451.

Ferrari, A., Licata, M., Rustichelli, C., Baraldi, C., Vandelli, D., Marchesi, F., Palazzoli, F., Verri, P. and Silingardi, E., 2017. Monitoring of adherence to headache treatments by means of hair analysis. *European Journal of Clinical Pharmacology*, *73*(2), pp.197-203.

Fingerhut, R., Silva Polanco, M.L., Arevalo, S., De Jesus, G. and Swiderska, M.A., 2014. First experience with a fully automated extraction system for simultaneous on-line direct tandem mass spectrometric analysis of amino acids and (acyl-) carnitines in a newborn screening setting. *Rapid Communications in Mass Spectrometry*, *28*(8), pp.965-973.

Flesch, G., Müller, P. and Lloyd, P., 1997. Absolute bioavailability and pharmacokinetics of valsartan, an angiotensin II receptor antagonist, in man. *European Journal of Clinical Pharmacology*, *52*(2), pp.115-120.

Flores, G.L., Cruz, H.M., Potsch, D.V., May, S.B., Brandão-Mello, C.E., Pires, M.M.A., Pilotto, J.H., Lewis-Ximenez, L.L., Lampe, E. and Villar, L.M., 2017. Evaluation of HBsAg

and anti-HBc assays in saliva and dried blood spot samples according HIV status. *Journal of Virological Methods*, *247*, pp.32-37.

Forrisier, J. and Firlick, F., 2014. Estimated Annual Pharmaceutical Revenue Loss Due to Medication Non-Adherence, https://adherence564.com/, (accessed 15.04.17).

Gajula, R., Pilli, N.R., Ravi, V.B., Maddela, R., Inamadugu, J.K., Polagani, S.R. and Busa, S., 2012. Simultaneous determination of atorvastatin and aspirin in human plasma by LC–MS/MS: its pharmacokinetic application. *Scientia Pharmaceutica*, *80*(4), pp.923-940.

Garcia, M.J., Reinoso, R.F., Sanchez Navarro, A. and Prous, J.R., 2003. Clinical pharmacokinetics of statins. *Methods and Findings Experimental Clinical Pharmacology*, *25*(6), pp.457-481.

Ghosh, C., Jain, I., Gaur, S., Patel, N., Upadhyay, A. and Chakraborty, B.S., 2011. Simultaneous estimation of atorvastatin and its two metabolites from human plasma by ESI-LC-MS/MS. *Drug Testing and Analysis*, *3*(6), pp.352-362.

Gjelstad, A. and Pedersen-Bjergaard, S., 2014. Challenges and new directions in analytical sample preparation. *Analytical and Bioanalytical Chemistry, 406*, pp.375–376

Goldstein, C.M., Gathright, E.C. and Garcia, S., 2017. Relationship between depression and medication adherence in cardiovascular disease: the perfect challenge for the integrated care team. *Patient Preference and Adherence*, *11*, pp.547 – 559.

Gomez, H.J., Cirillo, V.J. and Moncloa, F., 1987. The clinical pharmacology of lisinopril. *Journal of Cardiovascular Pharmacology*, *9*, pp.S27-S34.

Gonzalez, O., Alonso, R.M., Ferreirós, N., Weinmann, W., Zimmermann, R. and Dresen, S., 2011. Development of an LC–MS/MS method for the quantitation of 55 compounds prescribed in combined cardiovascular therapy. *Journal of Chromatography B*, *879*(3), pp.243-252.

González, O., Ferreirós, N., Blanco, M.E. and Alonso, R.M., 2015. Cardiovascular drug determination in bioanalysis: an update. *Bioanalysis*, *7*(18), pp.2399-2417.

Gonzalez, O., Iriarte, G., Rico, E., Ferreirós, N., Maguregui, M.I., Alonso, R.M. and Jiménez, R.M., 2010. LC–MS/MS method for the determination of several drugs used in combined cardiovascular therapy in human plasma. *Journal of Chromatography B*, *878*(28), pp.2685-2692.

Granger, B.B., Ekman, I., Hernandez, A.F., Sawyer, T., Bowers, M.T., DeWald, T.A., Zhao, Y., Levy, J. and Bosworth, H.B., 2015. Results of the chronic heart failure intervention to improve medication adherence study: a randomized intervention in high-risk patients. *American Heart Journal*, *169*(4), pp.539-548.

Green, M.D., Mount, D.L. and Nettey, H., 2002. High-performance liquid chromatographic assay for the simultaneous determination of sulfadoxine and pyrimethamine from whole blood dried onto filter paper. *Journal of Chromatography B*, *767*(1), pp.159-162.

Griffiths, R.L., Dexter, A., Creese, A.J. and Cooper, H.J., 2015. Liquid extraction surface analysis field asymmetric waveform ion mobility spectrometry mass spectrometry for the analysis of dried blood spots. *Analyst*, *140*(20), pp.6879-6885.

Guidance for industry: bioanalytical method validation, US Department of Health and Human Services, Food and Drugs Administration, 2001. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf/, (accessed 15.07.16).

Guideline on bioanalytical method validation, European Medicine Agency, 2011. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf/, (accessed 15.07.15).

Gupta, P., Patel, P., Horne, R., Buchanan, H., Williams, B. and Tomaszewski, M., 2016. How to screen for non-adherence to antihypertensive therapy. *Current Hypertension Reports*, *18*(12), pp.89-97

Gupta, S. and Bhatia, S., 2017. Optimising medication adherence in children with cancer. *Current Opinion in Pediatrics*, *29*(1), pp.41-45

Gupta, V.K., Jain, R., Lukram, O., Agarwal, S. and Dwivedi, A., 2011. Simultaneous determination of ramipril, ramiprilat and telmisartan in human plasma using liquid chromatography tandem mass spectrometry. *Talanta*, *83*(3), pp.709-716.

Hafezi, H., Robertson, T.L., Moon, G.D., Au-Yeung, K.Y., Zdeblick, M.J. and Savage, G.M., 2015. An ingestible sensor for measuring medication adherence. *IEEE Transactions on Biomedical Engineering*, *62*(1), pp.99-109.

Halladay, J.R., Donahue, K.E., Cené, C.W., Li, Q., Cummings, D.M., Hinderliter, A.L., Miller, C.L., Garcia, B.A., Little, E., Rachide, M. and Tillman, J., 2017. The association of health literacy and blood pressure reduction in a cohort of patients with hypertension. *Patient Education and Counseling*, *100*(3), pp.542-549.

Hamdidouche, I., Jullien, V., Billaud, E.M., Boutouyrie, P., Azizi, M. and Laurent, S., 2015. 7B. 06: routine urinary detection of antihypertensive drugs for estimation of adherence to treatment: a cross sectional study. *Journal of Hypertension*, *33*, pp.e93.

Hamdidouche, I., Jullien, V., Boutouyrie, P., Billaud, E., Azizi, M. and Laurent, S., 2017. Routine urinary detection of antihypertensive drugs for systematic evaluation of adherence to treatment in hypertensive patients. *Journal of Hypertension*, *35*(9), pp.1891-1898.

Hameed, M.A., Tebbit, L., Jacques, N., Thomas, M. and Dasgupta, I., 2015. Non-adherence to antihypertensive medication is very common among resistant hypertensives: results of a directly observed therapy clinic. *Journal of Human Hypertension*, *30*(2), pp.83 - 89

Hannon, W.H., Whitley, R.J., Davin, B., Fernhoff, P., Halonen, T., Lavochkin, M., Miller, J., Ojodu, J. and Therrell, B.L., 2007. Blood collection on filter paper for newborn screening programs; approved standard. *Clinical and Laboratory Standard Institute*, *23*(21), pp.1-33.

Harris, K., Mosler, G., Williams, S. and Grigg, J., 2016. Suboptimal asthma control and asthma medication adherence in UK secondary school children. *American Journal of Respiratory and Critical Care Medicine*, 193, pp.2162

Hawwa, A.F., AlBawab, A., Rooney, M., Wedderburn, L.R., Beresford, M.W. and McElnay, J.C., 2014. A novel dried blood spot-LCMS method for the quantification of methotrexate polyglutamates as a potential marker for methotrexate use in children. *PloS One*, *9*(2), pp.e89908.

Haynes, R.B., Taylor, D.W., Sackett, D.L., Gibson, E.S., Bernholz, C.D. and Mukherjee, J., 2016. Can simple clinical measurements detect patient noncompliance? *Hypertension*, *2*(6), pp.757-764.

Hazell, B. and Robson, R., 2015. Pharmaceutical waste reduction in the NHS. *NHS England Report, Version 1*.

Health & Social Care Information Centre, prescription cost analysis 2014 https://digital.nhs.uk/catalogue/PUB17274, (assessed 30.04.15).

Heinig, K., Wirz, T., Bucheli, F. and Gajate-Perez, A., 2011. Determination of oseltamivir (Tamiflu) and oseltamivir carboxylate in dried blood spots using offline or online extraction. *Bioanalysis*, *3*(4), pp.421-437.

HemaSpot-HF blood collection device, 2012. https://www.spotonsciences.com/products/hemaspot-hf/, (accessed 06.09.17).

HemaXis-DB blood collection device, 2017. http://hemaxis.com/technology/advanced microfluidics for blood sampling, (accessed 06.11.17).

Henion, J., Oliveira, R., Fumin, L., Foley, T. and Pomponio, R., 2013. Dried blood spots: the future. *Microsampling in Pharmaceutical Bioanalysis,* Future Science Ltd, pp48-66.

Henry, H., Sobhi, H.R., Scheibner, O., Bromirski, M., Nimkar, S.B. and Rochat, B., 2012. Comparison between a high-resolution single-stage Orbitrap and a triple quadrupole mass spectrometer for quantitative analyses of drugs. *Rapid Communications in Mass Spectrometry*, *26*(5), pp.499-509.

Hermann, P.H., Rodger, S.D., Remones, G., Thenot, J.P., London, D.R. and Morselli, P.L., 1983. Pharmacokinetics of diltiazem after intravenous and oral administration. *European Journal of Clinical Pharmacology*, *24*(3), pp.349-352.

Hettich Laboratory Application note, determination of the hematocrit value by centrifugation. http://www.hettichlab.com/documents/22402/225be2b3-ef94-487b-a4b3-532e0c766f40/, (accessed 12.09.16).

Heussner, K., Rauh, M., Cordasic, N., Menendez-Castro, C., Huebner, H., Ruebner, M., Schmidt, M., Hartner, A., Rascher, W. and Fahlbusch, F.B., 2017. Adhesive blood microsampling systems for steroid measurement via LC–MS/MS in the rat. *Steroids*, *120*, pp.1-6.

Ho, P.M., Bryson, C.L. and Rumsfeld, J.S., 2009. Medication adherence its importance in cardiovascular outcomes. *Circulation*, *119*(23), pp.3028-3035.

Ho, P.M., Magid, D.J., Shetterly, S.M., Olson, K.L., Maddox, T.M., Peterson, P.N., Masoudi, F.A. and Rumsfeld, J.S., 2008. Medication nonadherence is associated with a broad range of adverse outcomes in patients with coronary artery disease. *American Heart Journal*, *155*(4), pp.772-779.

Hoffman, J.T., Rossi, S.S., Espina-Quinto, R., Letendre, S. and Capparelli, E.V., 2013. Determination of Efavirenz in Human Dried Blood Spots by Reversed-Phase High Performance Liquid Chromatography with UV Detection. *Therapeutic Drug Monitoring*, *35*(2), pp. 143-152.

Horne, R., Weinman, J., Barber, N., Elliott, R. and Morgan, M., 2014. Concordance, adherence and compliance in medicine taking: report for the National Co-ordinating Centre for NHS Delivery and Organisation R & D (NCCSDO), pp.40-46.

Hosie, J. and Meredith, P., 1991. The pharmacokinetics of ramipril in a group of ten elderly patients with essential hypertension. *Journal of Cardiovascular Pharmacology*, *18*, pp.S125-S127.

Hotha, K.K., Roychowdhury, S., Mullangi, R. and Ravindranath, L.K., 2013. Rapid quantification of amlodipine enantiomers in human plasma by LC-MS/MS: application to a clinical pharmacokinetic study. *Biomedical Chromatography*, *27*(9), pp.1192-1199.

Huang, J., Xu, Y., Liu, F., Gao, S. and Guo, Q., 2006. Development of a liquid chromatography/tandem mass spectrometry assay for the quantification of lisinopril in human plasma. *Rapid Communications in Mass Spectrometry*, *20*(2), pp.248-252.

Huang, M.Q., Lin, Z. and Weng, N., 2013. Applications of high-resolution MS in bioanalysis. *Bioanalysis*, *5*(10), pp.1269-1276.

IMS Institute for Healthcare Informatics. Avoidable costs in U.S. health care: the $200 billion opportunity from using medicines more responsibly, 2013. Available at: http://www.imshealth.com/deployedfiles/imshealth/Global/Content/Corporate/IMS%20Institute/RUO -2013/IHII_Responsible Use_Medicines_2013.pdf, (accessed 07.05.14).

Instant Chemoinformatic Solutions, http://www.chemicalize.org, 2017 (assessed 13.06.17).

Iqbal, M., Khuroo, A., Batolar, L.S., Tandon, M., Monif, T. and Sharma, P.L., 2010. Pharmacokinetics and bioequivalence study of three oral formulations of valsartan 160 mg: a single-dose, randomized, open-label, three-period crossover comparison in healthy Indian male volunteers. *Clinical Therapeutics*, *32*(3), pp.588-596.

Irshaid, Y.M., Rawashdeh, N.M., Awwadi, F.F. and Kato, M.K., 1996. Comparative pharmacokinetics of two brands of atenolol following a single oral administration. *International Journal of Clinical Pharmacology and Therapeutics*, *34*(10), pp.457-461.

Islam, M.R., Mahdi, J.G. and Bowen, I.D., 1997. Pharmacological importance of stereochemical resolution of enantiomeric drugs. *Drug Safety*, *17*(3), pp.149-165.

Iuga, A.O. and McGuire, M.J., 2014. Adherence and health care costs. *Risk Management and Healthcare Policy*, *7*, p.35.

Iwasa, T., Takano, T., Hara, K.I. and Kamei, T., 1999. Method for the simultaneous determination of losartan and its major metabolite, EXP-3174, in human plasma by liquid chromatography–electrospray ionisation tandem mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications*, *734*(2), pp.325-330.

Jackevicius, C.A., Li, P. and Tu, J.V., 2008. Prevalence, predictors, and outcomes of primary nonadherence after acute myocardial infarction. *Circulation*, *117*(8), pp.1028-1036.

Jang, S.B., Lee, Y.J., Lim, L.A., Park, K.M., Kwon, B.J., Woo, J.S., Kim, Y.I., Park, M.S., Kim, K.H. and Park, K., 2010. Pharmacokinetic comparison of controlled-release and immediate-release oral formulations of simvastatin in healthy Korean subjects: a randomized, open-label, parallel-group, single-and multiple-dose study. *Clinical Therapeutics*, *32*(1), pp.206-216.

Jangala, H., Vats, P., Khuroo, A.H. and Monif, T., 2014. Development and validation of a LC-MS/MS method for the simultaneous estimation of amlodipine and valsartan in human plasma: Application to a bioequivalence study. *Scientia Pharmaceutica*, *82*(3), pp.585-600.

Jemal, M. and Xia, Y.Q., 2000. Bioanalytical method validation design for the simultaneous quantitation of analytes that may undergo interconversion during analysis. *Journal of Pharmaceutical and Biomedical Analysis*, *22*(5), pp.813-827.

Jemal, M., Ouyang, Z. and Powell, M.L., 2000. Direct-injection LC–MS–MS method for high-throughput simultaneous quantitation of simvastatin and simvastatin acid in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, *23*(2), pp.323-340.

Ji, H.Y., Park, E.J., Lee, K.C. and Lee, H.S., 2008. Quantification of doxazosin in human plasma using hydrophilic interaction liquid chromatography with tandem mass spectrometry. *Journal of Separation Science*, *31*(9), pp.1628-1633.

Jia, J.Y., Zhang, M.Q., Liu, Y.M., Liu, Y., Liu, G.Y., Li, S.J., Lu, C., Weng, L.P., Qi, Y.L. and Yu, C., 2010. Pharmacokinetics and bioequivalence evaluation of two losartan potassium 50-mg tablets: a single-dose, randomized-sequence, open-label, two-way crossover study in healthy Chinese male volunteers. *Clinical Therapeutics*, *32*(7), pp.1387-1395.

Johnson, J.V., Yost, R.A., Kelley, P.E. and Bradford, D.C., 1990. Tandem-in-space and tandem-in-time mass spectrometry: triple quadrupoles and quadrupole ion traps. *Analytical Chemistry*, *62*(20), pp.2162-2172.

Jonsson, O., Villar, R.P., Nilsson, L.B., Norsten-Höög, C., Brogren, J., Eriksson, M., Königsson, K. and Samuelsson, A., 2012. Capillary microsampling of 25 µl blood for the determination of toxicokinetic parameters in regulatory studies in animals. *Bioanalysis*, *4*(6), pp.661-674.

Joseph, P.G., Pare, G., Ross, S., Roberts, R. and Anand, S.S., 2014. Pharmacogenetics in Cardiovascular Disease: The Challenge of Moving from Promise to Realization. *Clinical Cardiology*, *37*(1), pp.48-56.

Kailasam, S. 2011. Simultaneous determination of therapeutic drug analytes in human plasma using LC/MS/MS. *Application Note, Agilent Technologies India Pvt*. Limited, Bangalore, India.

Kallem, R.R., Inamadugu, J.K., Ramesh, M. and Seshagirirao, J.V.L.N., 2012. Sensitive LC-MS/MS-ESI method for simultaneous determination of nifedipine and atenolol in human plasma and its application to a human pharmacokinetic study. *Biomedical Chromatography*, *27*(3), pp.349-355.

Kallem, R.R., Mullangi, R., Hotha, K.K., Ravindranath, L.K., Spoorthy, Y.N. and Seshagirirao, J.V.L.N., 2013. Simultaneous estimation of amlodipine and atenolol in human plasma: a sensitive LC–MS/MS method validation and its application to a clinical PK study. *Bioanalysis, 5*(7) pp.827-837.

Kardas, P., Lewek, P. and Matyjaszczyk, M., 2013. Determinants of patient adherence: a review of systematic reviews. *Frontiers in Pharmacology*, *4,* pp.91.

Kaufmann, A., Butcher, P., Maden, K., Walker, S. and Widmer, M., 2011. Quantitative and confirmative performance of liquid chromatography coupled to high-resolution mass spectrometry compared to tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, *25*(7), pp.979-992.

Keenan, J., 2017. Improving adherence to medication for secondary cardiovascular disease prevention. *European Journal of Preventive Cardiology*, *24*(3_suppl), pp.29-35.

Kelly, J.G. and O'Malley, K., 1990. Clinical pharmacokinetics of the newer ACE inhibitors. *Clinical Pharmacokinetics*, *19*(3), pp.177-196.

Khair, K., 2014. Compliance, concordance and adherence: what are we talking about? *Haemophilia*, *20*(5), pp.601-603.

Khalesi, S., Irwin, C. and Sun, J., 2017. Dietary Patterns, Nutrition Knowledge, Lifestyle, and Health-Related Quality of Life: Associations with Anti-Hypertension Medication Adherence in a Sample of Australian Adults. *High Blood Pressure and Cardiovascular Prevention*, pp.1-10.

Kim, J.E., Ki, M.H., Yoon, I.S., Cho, H.J., Kim, R.S., Kim, G.T. and Kim, D.D., 2014. Pharmacokinetic properties and bioequivalence of 2 formulations of valsartan 160-mg tablets: a randomized, single-dose, 2-period crossover study in healthy Korean male volunteers. *Clinical Therapeutics*, *36*(2), pp.273-279.

King, L., Kotian, A. and Jairaj, M., 2014. Introduction of a routine quan/qual approach into research DMPK: experiences and evolving strategies. *Bioanalysis*, *6*(24), pp.3337-3348.

Kip, A.E., Kiers, K.C., Rosing, H., Schellens, J.H.M., Beijnen, J.H. and Dorlo, T.P., 2017. Volumetric absorptive microsampling (VAMS) as an alternative to conventional dried blood spots in the quantification of miltefosine in dried blood samples. *Journal of Pharmaceutical and Bomedical Analysis*, *135*, pp.160-166.

Kita, K. and Mano, Y., 2017. Application of volumetric absorptive microsampling device for quantification of tacrolimus in human blood as a model drug of high blood cell partition. *Journal of Pharmaceutical and Biomedical Analysis*, *143,* pp.168–175.

Kneepkens, E.L., Pouw, M.F., Wolbink, G.J., Schaap, T., Nurmohamed, M.T., Vries, A., Rispens, T. and Bloem, K., 2017. Dried blood spots from finger prick facilitate

therapeutic drug monitoring of adalimumab and anti-adalimumab in patients with inflammatory diseases. *British Journal of Clinical Pharmacology*. *83*(11), pp.2474-2484.

Kole, P., Rao, A., Kurawattimath, V. and Mandlekar, S., 2017. LC–MS determination of triazolam and its hydroxy metabolites in mouse dried blood spots: application to transgenic mouse pharmacokinetic studies. *Bioanalysis*, *9*(13), pp.987-1000.

Koop, D.R., Bleyle, L.A., Munar, M., Cherala, G. and Al-Uzri, A., 2013. Analysis of tacrolimus and creatinine from a single dried blood spot using liquid chromatography tandem mass spectrometry. *Journal of Chromatography B*, *926*, pp.54-61.

Korfmacher, W. and Ramanathan, R., 2016. HRMS in DMPK. *Bioanalysis, 8*(16), pp.1635-1637.

Korfmacher, W., 2011. High-resolution mass spectrometry will dramatically change our drug-discovery bioanalysis procedures. *Bioanalysis. 3*(11), pp.1169-1171.

Kosek, V., Stránská, M., Fenclová, M., Ruml, T., Vítek, L. and Hajšlová, J., 2017. High resolution mass spectrometry based method applicable for a wide range of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors in blood serum including intermediates and products of the cholesterol biosynthetic pathway. *Journal of Chromatography A*, *1489*, pp.86-94.

Koseki, N., Kawashita, H., Hara, H., Niina, M., Tanaka, M., Kawai, R., Nagae, Y. and Masuda, N., 2007. Development and validation of a method for quantitative determination of valsartan in human plasma by liquid chromatography-tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, *43*(5), pp.1769-1774.

Kosoglou, T., Meyer, I., Veltri, E.P., Statkevich, P., Yang, B., Zhu, Y., Mellars, L., Maxwell, S.E., Patrick, J.E., Cutler, D.L. and Batra, V.K., 2002. Pharmacodynamic interaction between the new selective cholesterol absorption inhibitor ezetimibe and simvastatin. *British Journal of Clinical Pharmacology*, *54*(3), pp.309-319.

Koster, R.A., Alffenaar, J.W.C., Botma, R., Greijdanus, B., Touw, D.J., Uges, D.R. and Kosterink, J.G., 2015. What is the right blood hematocrit preparation procedure for standards and quality control samples for dried blood spot analysis? *Bioanalysis. 7*(3), pp.345-351.

Koster, R.A., Alffenaar, J.W.C., Greijdanus, B. and Uges, D.R., 2013. Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. *Talanta*, *115*, pp.47-54.

Koster, R.A., Dijkers, E.C. and Uges, D.R., 2009. Robust, high-throughput LC-MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus, everolimus, and sirolimus in whole blood. *Therapeutic Drug Monitoring*, *31*(1), pp.116-125.

Koster, R.A., Veenhof, H., Botma, R., Hoekstra, A.T., Berger, S.P., Bakker, S.J., Alffenaar, J.W.C. and Touw, D.J., 2017. Dried blood spot validation of five immunosuppressants, without hematocrit correction, on two LC–MS/MS systems. *Bioanalysis, 9*(7), pp.1-11.

Koytchev, R., Ozalp, Y., Erenmemisoglu, A., Van der Meer, M.J. and Alpan, R.S., 2004. Bio equivalence study of atorvastatin tablets. *Arzneimittelforschung*, *54*(9A), pp.573-577.

Krauss, M., Singer, H. and Hollender, J., 2010. LC–high resolution MS in environmental analysis: from target screening to the identification of unknowns. *Analytical and Bioanalytical Chemistry*, *397*(3), pp.943-951.

Kronish, I., Woodward, M. and Sergie, Z., 2011. Meta-Analysis Impact of Drug Class on Adherence to Antihypertensives. *Circulation*, *123*, pp.1611-1621.

Kronish, I.M. and Ye, S., 2013. Adherence to cardiovascular medications: lessons learned and future directions. *Progress in Cardiovascular Diseases*, *55*(6), pp.590-600.

Kvedar, J.C., Nesbitt, T., Kvedar, J.G. and Darkins, A., 2011. E-patient connectivity and the near-term future. *Journal of General Internal Medicine*, *26*(2), pp.636-638.

Kwon, Y.H., Gwak, H.S., Yoon, S.J. and Chun, I.K., 2007. Pharmacokinetics of doxazosin gastrointestinal therapeutic system after multiple administration in Korean healthy volunteers. *Drug Development and Industrial Pharmacy*, *33*(8), pp.824-829.

La Caze, A., Gujral, G. and Cottrell, W.N., 2014. How do we better translate adherence research into improvements in patient care? *International Journal of Clinical Pharmacy*, *36*(1), pp.10-14.

La Marca, G., Giocaliere, E., Villanelli, F., Malvagia, S., Funghini, S., Ombrone, D., Filippi, L., De Gaudio, M., De Martino, M. and Galli, L., 2012. Development of an UPLC–MS/MS method for the determination of antibiotic ertapenem on dried blood spots. *Journal of Pharmaceutical and Biomedical Analysis*, *61*, pp.108-113.

Lancaster S.G. and Sorkin, E.M., 1998. Bisoprolol. A preliminary review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in hypertension and angina pectoris. *Drugs, 36*(3), pp.256-285.

Lancaster, S.G. and Todd, P.A., 1988. Lisinopril. *Drugs, 35*(6), pp.646-669.

Larsen, J., Stovring, H., Kragstrup, J. and Hansen, D.G., 2009. Can differences in medical drug compliance between European countries be explained by social factors: analyses based on data from the European Social Survey, round 2. *BMC Public Health*, *9*(1), pp.145.

Lawrence, D., Miller, J.H. and W Flexner, C., 2017. Medication Adherence. *The Journal of Clinical Pharmacology*, *57*(4), pp.422-427.

Lawson, A.J., Shipman, K.E., George, S. and Dasgupta, I., 2016. A Novel 'Dilute-and-Shoot' Liquid Chromatography–Tandem Mass Spectrometry Method for the Screening of Antihypertensive Drugs in Urine. *Journal of Analytical Toxicology*, *40*(1), pp.17-27.

Lawson, G., Cocks, E. and Tanna, S., 2012. Quantitative determination of atenolol in dried blood spot samples by LC–HRMS: a potential method for assessing medication adherence. *Journal of Chromatography B*, *897*, pp.72-79.

Lawson, G., Cocks, E. and Tanna, S., 2013. Bisoprolol, ramipril and simvastatin determination in dried blood spot samples using LC–HRMS for assessing medication adherence. *Journal of Pharmaceutical and Biomedical Analysis*, *81*, pp.99-107.

Lawson, G., Mulla, H. and Tanna, S., 2012. Captopril Determination in Dried Blood Spot Samples with LC-MS and LCHRMS: A Potential Method for Neonate Pharmacokinetic Studies. *Journal of Bioanalysis and Biomedicine, 4*(2), pp.016-025.

Lee, G.K., Wang, H.H., Liu, K.Q., Cheung, Y., Morisky, D.E. and Wong, M.C., 2013. Determinants of medication adherence to antihypertensive medications among a Chinese population using Morisky Medication Adherence Scale. *PloS One*, *8*(4), pp.e62775.

Lehmann, A., Aslani, P., Ahmed, R., Celio, J., Gauchet, A., Bedouch, P., Bugnon, O., Allenet, B. and Schneider, M.P., 2014. Assessing medication adherence: options to consider. *International Journal of Clinical Pharmacy*, *36*(1), pp.55-69.

Leicestershire health community NHS, medicines formulary. https://leicestershire.formulary.co.uk/chaptersSubDetails.asp?FormularySectionID=2&SubSectionRef=02.01&SubSectionID=A100, (assessed 30.10.14).

Lennernas, H., 2003. Clinical pharmacokinetics of atorvastatin. *Clinical Pharmacokinetics*, *42*(13), pp.1141-1160.

Leopold, G., 1986. Balanced pharmacokinetics and metabolism of bisoprolol. *Journal of Cardiovascular Pharmacology*, *8*, pp.S16-S20.

Leopold, G., Ungethum, W., Pabst, J., Simane, Z., Buhring, K.U. and Wiemann, H., 1986. Basic pharmacokinetics of bisoprolol, a new highly beta 1-selective adrenoceptor antagonist. *Journal of Clinical Pharmacology. 26*(8), pp. 616-621.

Leung, K.S.Y. and Fong, B.M.W., 2014. LC–MS/MS in the routine clinical laboratory: has its time come? *Analytical and Bioanalytical Chemistry*, *406*(9-10), pp.2289-2301.

Li, F., Zulkoski, J., Fast, D. and Michael, S., 2011. Perforated dried blood spots: a novel format for accurate microsampling. *Bioanalysis, 3*(20), pp.2321-2333.

Li, H., Wang, Y., Jiang, Y., Tang, Y., Wang, J., Zhao, L. and Gu, J., 2007. A liquid chromatography/tandem mass spectrometry method for the simultaneous quantification of valsartan and hydrochlorothiazide in human plasma. *Journal of Chromatography B*, *852*(1), pp.436-442.

Li, P.K., Lee, J.T., Conboy, K.A. and Ellis, E.F., 1986. Fluorescence polarization immunoassay for theophylline modified for use with dried blood spots on filter paper. *Clinical Chemistry*, *32*(3), pp.552-555.

Li, S., Liu, G., Jia, J., Liu, Y., Pan, C., Yu, C., Cai, Y. and Ren, J., 2007. Simultaneous determination of ten antiarrhythic drugs and a metabolite in human plasma by liquid chromatography—tandem mass spectrometry. *Journal of Chromatography B*, *847*(2), pp.174-181.

Li, W. and Lee, M.S. eds., 2014. *Dried Blood Spots: Applications and Techniques*. John Wiley & Sons, pp.3-76

Li, W. and Tse, F.L., 2010. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomedical Chromatography*, *24*(1), pp.49-65.

Li, Y., Henion, J., Abbott, R. and Wang, P., 2011. Dried blood spots as a sampling technique for the quantitative determination of guanfacine in clinical studies. *Bioanalysis, 3*(22), pp.2501-2514.

Linder, C., Wide, K., Walander, M., Beck, O., Gustafsson, L.L. and Pohanka, A., 2016. Comparison between dried blood spot and plasma sampling for therapeutic drug monitoring of antiepileptic drugs in children with epilepsy: A step towards home sampling. *Clinical Biochemistry*, *50*(7-8), pp.418-424.

Liu, F., Zhang, J., Xu, Y., Gao, S. and Guo, Q., 2008. Simultaneous determination of hydrochlorothiazide and valsartan in human plasma by liquid chromatography/tandem mass spectrometry. *Analytical Letters*, *41*(8), pp.1348-1365.

Liu, K., Zhong, D. and Chen, X., 2010. Enantioselective determination of doxazosin in human plasma by liquid chromatography–tandem mass spectrometry using

ovomucoid chiral stationary phase. *Journal of Chromatography B*, *878*(26), pp.2415-2420.

Liu, L., Wen, Y., Liu, K., Sun, L., Lu, Y. and Yin, Z., 2014. Simultaneous determination of a broad range of cardiovascular drugs in plasma with a simple and efficient extraction/clean up procedure and chromatography-mass spectrometry analysis. *RSC Advances*, *4*(38), pp.19629-19639.

Liu, M., Zhang, D., Sun, Y., Wang, Y., Liu, Z. and Gu, J., 2007. Liquid chromatographic–electrospray tandem mass spectrometric determination of bisoprolol in human plasma. *Biomedical Chromatography*, *21*(5), pp.508-513.

Lu, X.Y., Shen-Tu, J.Z. and Liu, J., 2006. High-performance liquid chromatography–mass spectrometric analysis of ramipril and its active metabolite ramiprilat in human serum: Application to a pharmacokinetic study in the Chinese volunteers. *Journal of Pharmaceutical and Biomedical Analysis*, *40*(2), pp.478-483.

Lv, C.M., Wei, C.M., Bu, F.L., Chen, R., Wang, X.L., Li, R., Wang, B.J. and Guo, R.C., 2013. Determination of amlodipine in human plasma by LC-MS/MS and its bioequivalence study in healthy Chinese subjects. *Pharmacology & Pharmacy*, *4*(02), pp.191-200.

Lynch, K.L., 2016. CLSI C62-A: a new standard for clinical mass spectrometry. *Clinical Chemistry*, *62*(1), pp.24-29.

Ma, N., Liu, W., Li, H., Chen, B., Zhu, Y., Liu, X., Wang, F., Xiang, D. and Zhang, B., 2006. LC–MS determination and relative bioavailability of doxazosin mesylate tablets in healthy Chinese male volunteers. *Journal of Pharmaceutical and Biomedical Analysis*, *43*(3), pp.1049-1056.

Ma, S. and Chowdhury, S.K., 2013. Data acquisition and data mining techniques for metabolite identification using LC coupled to high-resolution MS. *Bioanalysis*, 5(10), pp.1285-1297.

MacLaughlin, E.J., Raehl, C.L., Treadway, A.K., Sterling, T.L., Zoller, D.P. and Bond, C.A., 2005. Assessing medication adherence in the elderly. *Drugs & Aging*, *22*(3), pp.231-255.

Magiera, S., Baranowska, I. and Kusa, J., 2012. Development and validation of UHPLC–ESI-MS/MS method for the determination of selected cardiovascular drugs, polyphenols and their metabolites in human urine. *Talanta*, *89*, pp.47-56.

Mahoney, J.J., Ansell, B.J., Fleming, W.K. and Butterworth, S.W., 2008. The unhidden cost of noncompliance. *Journal of Managed Care Pharmacy*, *14*(6 Supp B), pp.1-30.

Malek, N., Heath, C.A. and Greene, J., 2017. A review of medication adherence in people with epilepsy. *Acta Neurologica Scandinavica*, *135*(5), pp.507-515.

Manicke, N.E., Bills, B.J. and Zhang, C., 2016. Analysis of biofluids by paper spray MS: advances and challenges. *Bioanalysis*, *8*(6), pp.589-606.

Mano, Y., Kita, K. and Kusano, K., 2015. Hematocrit-independent recovery is a key for bioanalysis using volumetric absorptive microsampling devices, Mitra™. *Bioanalysis*, *7*(15), pp.1821-1829.

Martial, L.C., Aarnoutse, R.E., Schreuder, M.F., Henriet, S.S., Brüggemann, R.J. and Joore, M.A., 2016. Cost Evaluation of Dried Blood Spot Home Sampling as Compared to Conventional Sampling for Therapeutic Drug Monitoring in Children. *PloS One*, *11*(12), p.e0167433.

Martial, L.C., Hoogtanders, K.E., Schreuder, M.F., Cornelissen, E.A., van der Heijden, J., Joore, M.A., Van Maarseveen, E.M., Burger, D.M., Croes, S., Brüggemann, R.J. and Aarnoutse, R.E., 2017. Dried Blood Spot Sampling for Tacrolimus and Mycophenolic Acid in Children: Analytical and Clinical Validation. *Therapeutic Drug Monitoring*, *39*(4), pp.412-421.

Martinez, J. and Finken, G., 2017. Improved Medication Adherence with Pharmacist Interventions for Patients with Diabetes in Phase 3 NIGLM Clinical Trials. In *Diabetes* (66), pp. A178-A179).

Massaroti, P., Marchioretto, M.A.M., Cassiano, N.M., Bernasconi, G., Calafatti, S.A., Barros, F.A.P., Meurer, E.C. and Pedrazzoli, J., 2005. Development and validation of a selective and robust LC-MS/MS method for quantifying amlodipine in human plasma. *Analytical and Bioanalytical Chemistry*, *382*(4), pp.1049-1054.

Mathes, T., Antoine, S.L., Pieper, D. and Eikermann, M., 2014. Adherence enhancing interventions for oral anticancer agents: a systematic review. *Cancer Treatment Reviews*, *40*(1), pp.102-108.

Mathes, T., Jaschinski, T. and Pieper, D., 2014. Adherence influencing factors–a systematic review of systematic reviews. *Archives of Public Health*, *72*(1), pp.37-53.

Mayo-Gamble, T.L. and Mouton, C., 2017. Examining the association between health literacy and medication adherence among older adults. *Health Communication*, pp.1-7.

McCarthy, R., 1998. The price you pay for the drug not taken. *Business and Health*, *16*(10), p.27.

Medication nonadherence, a $300B+ healthcare problem, (2017) https://www.adherium.com/,(assessed 30.10.17).

Meesters, R.J., Zhang, J., van Huizen, N.A., Hooff, G.P., Gruters, R.A. and Luider, T.M., 2012. Dried matrix on paper disks: the next generation DBS microsampling technique for managing the hematocrit effect in DBS analysis. *Bioanalysis*, *4*(16), pp.2027-2035.

Meisel, S., Shamiss, A. and Rosenthal, T., 1994. Clinical pharmacokinetics of ramipril. *Clinical Pharmacokinetics*, *26*(1), pp.7-15.

Mendoza, L., Hajduch, M., Plaušinaitis, R., Platilova, V., Emritte, N. and Svoboda, M., 2006. Pharmacokinetic and bioequivalence testing of atorvastatin formulations in healthy male volunteers. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *61*(9), pp.805-806.

Meredith, P.A. and Elliott, H.L., 1992. Clinical pharmacokinetics of amlodipine. *Clinical Pharmacokinetics*, *22*(1), pp.22-31.

Midha, K.K., McKay, G., Rawson, M.J. and Hubbard, J.W., 1998. The impact of stereoisomerism in bioequivalence studies. *Journal of Pharmaceutical Sciences*, *87*(7), pp.797-802.

Milosheska, D., Grabnar, I. and Vovk, T., 2015. Dried blood spots for monitoring and individualization of antiepileptic drug treatment. *European Journal of Pharmaceutical Sciences*, *75*, pp.25-39.

Mislang, A.R., Wildes, T.M., Kanesvaran, R., Baldini, C., Holmes, H.M., Nightingale, G., Coolbrandt, A. and Biganzoli, L., 2017. Adherence to oral cancer therapy in older adults: The International Society of Geriatric Oncology (SIOG) taskforce recommendations. *Cancer Treatment Reviews, 57,* pp.58-66.

Mitchell, G., Porter, S. and Manias, E., 2014. Adherence to oral chemotherapy: a review of the evidence. *Cancer Nursing Practice*, *13*(4), pp.25-28.

Mitra microsampling device, 2014. https://www.neoteryx.com/microsampling-collection-devices, (accessed 30.08.17).

Moffat, A.C., Osselton, M.D., Widdop, B. and Watts, J., 2011. *Clarke's analysis of drugs and poisons in pharmaceuticals, body fluids and post mortem materials* (Vol. 3). 4th edition, London: Pharmaceutical press, pp.69 -73

Mohan, S.J., Mohan, E.C. and Yamsani, M.R., 2009. Chirality and its importance in pharmaceutical field-an overview. *International Journal of Pharmaceutical Sciences and Nanotechnology*, *1*(4), pp.309-316.

Molden, E., Boe, G.H., Christensen, H. and Reubsaet, L., 2003. High-performance liquid chromatography–mass spectrometry analysis of diltiazem and 11 of its phase I metabolites in human plasma. *Journal of Pharmaceutical and Biomedical analysis*, *33*(2), pp.275-285.

Monroe, A.K., Pena, J.S., Moore, R.D., Riekert, K.A., Eakin, M.N., Kripalani, S. and Chander, G., 2017. Randomized controlled trial of a pictorial aid intervention for

medication adherence among HIV-positive patients with comorbid diabetes or hypertension. *AIDS Care*, pp.1-8.

Montouris, G. and Hohler, A.D., 2016. Cultural barriers to medication adherence in epilepsy. *CONTINUUM: Lifelong Learning in Neurology*, *22*(1), pp.266-269.

Moore, C., 2015. Drug testing and adherence monitoring in pain management: Oral fluid testing. *Journal of Opioid Management*, *11*(1), pp.69-75.

Morawski, K., Ghazinouri, R., Krumme, A., McDonough, J., Durfee, E., Oley, L., Mohta, N., Juusola, J. and Choudhry, N.K., 2017. Rationale and design of the Medication Adherence Improvement Support App for Engagement—Blood Pressure (MedISAFE-BP) trial. *American Heart Journal*, *186*, pp.40-47.

Morawski, K., Ghazinouri, R., Krumme, A., McDonough, J., Durfee, E., Oley, L., Mohta, N., Juusola, J. and Choudhry, N.K., 2017. Rationale and design of the Medication adherence Improvement Support App For Engagement—Blood Pressure (MedISAFE-BP) trial. *American Heart Journal*, *186*, pp.40-47.

Morrison, A., Stauffer, M.E. and Kaufman, A.S., 2015. Defining medication adherence in individual patients. *Patient Preference and Adherence*, *9*, pp.893-897.

Mukhtar, O., Weinman, J. and Jackson, S.H., 2014. Intentional non-adherence to medications by older adults. *Drugs & aging*, *31*(3), pp.149-157.

Murtaza, G., 2012. Solubility enhancement of simvastatin: a review. *Acta Poloniae Pharmaceutica*, *69*(4), pp.581-90.

Naderi, S.H., Bestwick, J.P. and Wald, D.S., 2012. Adherence to drugs that prevent cardiovascular disease: meta-analysis on 376,162 patients. *The American Journal of Medicine*, *125*(9), pp.882-887.

Najib, N.M., Idkaidek, N., Adel, A., Admour, I., Astigarraga, R.E., Nucci, G.D., Alam, S.M. and Dham, R., 2003. Pharmacokinetics and bioequivalence evaluation of two simvastatin 40 mg tablets (Simvast & Zocor) in healthy human volunteers. *Biopharmaceutics & Drug Disposition*, *24*(5), pp.183-189.

Najib, N.M., Idkaidek, N., Adel, A., Mohammed, B., Al-Masri, S., Admour, I., Alam, S.M. and Dham, R., 2005. Comparative bioavailability of two brands of atenolol 100 mg tablets (Tensotin and Tenormin) in healthy human volunteers. *Biopharmaceutics and Drug Disposition*, *26*(1), pp.1-5.

National Health Service, Guidelines for newborn blood spot sampling: quick reference guide, 2016. https://www.gov.uk/government/publications/newborn-blood-spot-screening-sampling-guidelines, (accessed 07.06.17).

National Institute for Clinical Excellence UK, Costing statement: Medication adherence. https://www.nice.org.uk/guidance/cg76/resources/costing-statement-242056477 (accessed 13.07.16).

National Institute for Clinical Excellence UK, Medicines adherence guidelines: involving patients in decisions about prescribed medicines and supporting adherence, 2009. https://www.nice.org.uk/guidance/cg76/chapter/introduction (accessed 14.03.14).

Neiheisel, M.B., Wheeler, K.J. and Roberts, M.E., 2014. Medication adherence part one: Understanding and assessing the problem. *Journal of the American Association of Nurse Practitioners*, *26*(1), pp.49-55.

Nieuwlaat, R., Wilczynski, N., Navarro, T., Hobson, N., Jeffery, R., Keepanasseril, A., Agoritsas, T., Mistry, N., Iorio, A., Jack, S. and Sivaramalingam, B., 2014. Interventions for enhancing medication adherence. *The Cochrane Library*, pp.001-732.

Nilsson, L.B., Ahnoff, M. and Jonsson, O., 2013. Capillary microsampling in the regulatory environment: validation and use of bioanalytical capillary microsampling methods. *Bioanalysis*, *5*(6), pp.731-738.

Nirogi, R., Mudigonda, K. and Kandikere, V., 2007. Chromatography–mass spectrometry methods for the quantitation of statins in biological samples. *Journal of Pharmaceutical and Biomedical Analysis*, *44*(2), pp.379-387.

Nirogi, R.V., Kandikere, V.N., Mudigonda, K., Shukla, M. and Maurya, S., 2006. Sensitive and rapid liquid chromatography/tandem mass spectrometry assay for the

quantification of amlodipine in human plasma. *Biomedical Chromatography*, *20*(9), pp.833-842.

Nobre, M.R.C. and Domingues, R.Z.D.L., 2017. Patient adherence to ischemic heart disease treatment. *Revista da Associação Médica Brasileira*, *63*(3), pp.252-260.

Nováková, L., Šatínský, D. and Solich, P., 2008. HPLC methods for the determination of simvastatin and atorvastatin. *TrAC Trends in Analytical Chemistry*, *27*(4), pp.352-367.

Nováková, L., Vlčková, H., Šatínský, D., Sadílek, P., Solichová, D., Bláha, M., Bláha, V. and Solich, P., 2009. Ultra-high-performance liquid chromatography tandem mass spectrometric detection in clinical analysis of simvastatin and atorvastatin. *Journal of Chromatography B*, *877*(22), pp.2093-2103.

Novilytic limited, Noviplex cards, next generation dried plasma spot technology, 2016. http://www.ssi.shimadzu.com/products/product.cfm?product=noviplex, (accessed 03.11.17).

Nys, G., Kok, M.G., Servais, A.C. and Fillet, M., 2017. Beyond dried blood spot: current microsampling techniques in the context of biomedical applications. *Trends in Analytical Chemistry*. 97, pp.326-332.

O'mara, M., Hudson-Curtis, B., Olson, K., Yueh, Y., Dunn, J. and Spooner, N., 2011. The effect of hematocrit and punch location on assay bias during quantitative bioanalysis of dried blood spot samples. *Bioanalysis*, *3*(20), pp.2335-2347.

Ochiai, H., Uchiyama, N., Imagaki, K., Hata, S. and Kamei, T., 1997. Determination of simvastatin and its active metabolite in human plasma by column-switching high-performance liquid chromatography with fluorescence detection after derivatization with 1-bromoacetylpyrene. *Journal of Chromatography B: Biomedical Sciences and Applications*, *694*(1), pp.211-217.

Ohtawa, M., Takayama, F., Saitoh, K., Yoshinaga, T. and Nakashima, M., 1993. Pharmacokinetics and biochemical efficacy after single and multiple oral

administration of losartan, an orally active nonpeptide angiotensin II receptor antagonist, in humans. *British Journal of Clinical Pharmacology*, *35*(3), pp.290-297.

Olds, P.K., Kiwanuka, J.P., Nansera, D., Huang, Y., Bacchetti, P., Jin, C., Gandhi, M. and Haberer, J.E., 2015. Assessment of HIV antiretroviral therapy adherence by measuring drug concentrations in hair among children in rural Uganda. *AIDS Care*, *27*(3), pp.327-332.

Oliveira, R.V., Henion, J. and Wickremsinhe, E., 2014. Fully-automated approach for online dried blood spot extraction and bioanalysis by two-dimensional-liquid chromatography coupled with high-resolution quadrupole time-of-flight mass spectrometry. *Analytical Chemistry*, *86*(2), pp.1246-1253.

Oliveira, R.V., Henion, J. and Wickremsinhe, E.R., 2014. Automated direct extraction and analysis of dried blood spots employing on-line SPE high-resolution accurate mass bioanalysis. *Bioanalysis*, *6*(15), pp.2027-2041.

Oliveira, R.V., Henion, J. and Wickremsinhe, E.R., 2014. Automated high-capacity on-line extraction and bioanalysis of dried blood spot samples using liquid chromatography/high-resolution accurate mass spectrometry. *Rapid Communications in Mass Spectrometry*, *28*(22), pp.2415-2426.

Osterberg, L. and Blaschke, T., 2005. Adherence to medication. *New England Journal of Medicine*, *353*(5), pp.487-497.

Otto, C.M., 2017. Heartbeat: Achieving better medication adherence. *Heart*, *103*(14), pp.1057-1058.

Oung, A.B., Kosirog, E., Chavez, B., Brunner, J. and Saseen, J.J., 2017. Evaluation of medication adherence in chronic disease at a federally qualified health center. *Therapeutic Advances in Chronic Disease*, *8*(8-9), pp.113-120.

Padua, A.A., Barrientos-Astigarraga, R.E., Rezende, V.M., Mendes, G.D. and De Nucci, G., 2004. Lisinopril quantification in human plasma by liquid chromatography–

electrospray tandem mass spectrometry. *Journal of Chromatography B*, *809*(2), pp.211-216.

Palmer, M.J., Barnard, S., Perel, P. and Free, C., 2017. Mobile phone-based interventions for improving adherence to medication prescribed for the primary prevention of cardiovascular disease in adults. *The Cochrane Library*, pp.1 – 12.

Pandya, K.K., Satia, M., Gandhi, T.P., Modi, I.A., Modi, R.I. and Chakravarthy, B.K., 1995. Detection and determination of total amlodipine by high-performance thin-layer chromatography: a useful technique for pharmacokinetic studies. *Journal of Chromatography B: Biomedical Sciences and Applications*, *667*(2), pp.315-320.

Park, J.Y., Kim, K.A., Park, P.W., Lee, O.J., Kim, J.S., Lee, G.H., Ha, M.C., Park, J.H. and Ryu, J.H., 2006. Comparative pharmacokinetic and pharmacodynamic characteristics of amlodipine besylate and amlodipine nicotinate in healthy subjects. *International Journal of Clinical Pharmacology and Therapeutics*, *44*(12), pp.641-647.

Park, L.G., Howie-Esquivel, J. and Dracup, K., 2015. Electronic measurement of medication adherence. *Western Journal of Nursing Research*, *37*(1), pp.28-49.

Parker, S.L., Dorofaeff, T., Lipman, J., Ballot, D.E., Bandini, R.M., Wallis, S.C. and Roberts, J.A., 2016. Is there a role for microsampling in antibiotic pharmacokinetic studies? *Expert opinion on Drug Metabolism & Toxicology*, *12*(6), pp.601-614.

Partani, P., Verma, S.M., Gurule, S., Khuroo, A. and Monif, T., 2014. Simultaneous quantitation of atorvastatin and its two active metabolites in human plasma by liquid chromatography/electrospray tandem mass spectrometry. *Journal of Pharmaceutical Analysis*, *4*(1), pp.26-36.

Partridge, A.H., Avorn, J., Wang, P.S. and Winer, E.P., 2002. Adherence to therapy with oral antineoplastic agents. *Journal of the National Cancer Institute*, *94*(9), pp.652-661.

Patel, B.N., Sharma, N., Sanyal, M. and Shrivastav, P.S., 2008. Simultaneous determination of simvastatin and simvastatin acid in human plasma by LC-MS/MS

without polarity switch: Application to a bioequivalence study. *Journal of Separation Science*, *31*(2), pp.301-313.

Patel, J.R., Pethani, T.M., Vachhani, A.N., Sheth, N.R. and Dudhrejiya, A.V., 2014. Development and validation of bioanalytical method for simultaneous estimation of ramipril and hydrochlorothiazide in human plasma using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B*, *970*, pp.53-59.

Patel, M. and Kothari, C., 2017. Critical review of statins: A bio-analytical perspective for therapeutic drug monitoring. *TrAC Trends in Analytical Chemistry*, *86*, pp.206-221.

Patel, P., 2011. An investigation into the use of dried blood spot analysis in pharmacokinetic studies. Doctoral Thesis, *De Montfort University, Leicester UK*. p 59.

Pelton, R., 2009. Bioactive paper provides a low-cost platform for diagnostics. *TrAC Trends in Analytical Chemistry*, *28*(8), pp.925-942.

Pérez-Escamilla, B., Franco-Trigo, L., Moullin, J.C., Martínez-Martínez, F. and García-Corpas, J.P., 2015. Identification of validated questionnaires to measure adherence to pharmacological antihypertensive treatments. *Patient Preference and Adherence*, *9*, pp.569-578.

Phan, S.V., 2016. Medication adherence in patients with schizophrenia. *The International Journal of Psychiatry in Medicine*, *51*(2), pp.211-219.

Pharmaceutical Group of the European Union, targeting adherence, http://www.pgeu.eu/en/component/policy/policy/110-policy/5-adherence.html?Itemid=101 (assessed 15.03.17)

Phyo Lwin, E.M., Gerber, C., Song, Y., Leggett, C., Ritchie, U., Turner, S. and Garg, S., 2017. A new LC–MS/MS bioanalytical method for atenolol in human plasma and milk. *Bioanalysis*, *9*(7), pp.517-530.

Piepoli, M.F., Hoes, A.W., Agewall, S., Albus, C., Brotons, C., Catapano, A.L., Cooney, M.T., Corrà, U., Cosyns, B., Deaton, C. and Graham, I., 2016. 2016 European Guidelines on cardiovascular disease prevention in clinical practice: developed with the special

contribution of the European Association for Cardiovascular Prevention & Rehabilitation (EACPR). *European Heart Journal*, *37*(29), pp.2315-2381.

Pilli, N.R., Inamadugu, J.K., Mullangi, R., Karra, V.K., Vaidya, J.R. and Seshagiri Rao, J.V.L.N., 2010. Simultaneous determination of atorvastatin, amlodipine, ramipril and benazepril in human plasma by LC-MS/MS and its application to a human pharmacokinetic study. *Biomedical Chromatography*, *25*(4), pp.439-449.

Pilli, N.R., Mullangi, R., Inamadugu, J.K., Nallapati, I.K. and Rao, J.V., 2012. Simultaneous determination of simvastatin, lovastatin and niacin in human plasma by LC-MS/MS and its application to a human pharmacokinetic study. *Biomedical Chromatography: BMC*, *26*(4), pp.476-484.

Piórkowska, E., Musijowski, J., Buś-Kwaśnik, K. and Rudzki, P.J., 2017. Is a deuterated internal standard appropriate for the reliable determination of olmesartan in human plasma? *Journal of Chromatography B*, *1040*, pp.53-59.

Polinko, M., Riffel, K., Song, H. and Lo, M.W., 2003. Simultaneous determination of losartan and EXP3174 in human plasma and urine utilizing liquid chromatography/tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical analysis*, *33*(1), pp.73-84.

Pound, P., Britten, N., Morgan, M., Yardley, L., Pope, C., Daker-White, G. and Campbell, R., 2005. Resisting medicines: a synthesis of qualitative studies of medicine taking. *Social Science & Medicine*, *61*(1), pp.133-155.

Prasaja, B., Sasongko, L., Harahap, Y., Lusthom, W. and Grigg, M., 2009. Simultaneous quantification of losartan and active metabolite in human plasma by liquid chromatography–tandem mass spectrometry using irbesartan as internal standard. *Journal of Pharmaceutical and Biomedical Analysis*, *49*(3), pp.862-867.

Provatas, A.A., King, C.A., Kolakowski, S.L., Stuart, J.D. and Perkins, C.R., 2017. Quantification of Polycyclic Aromatic Hydrocarbons in Avian Dried Blood Spots by High-Performance Liquid Chromatography with Simple Liquid Extraction and Phospholipid Solid-Phase Extraction Preparation. *Analytical Letters*, *50*(16), pp.2649-2660.

Qi, W., Zhao, Q., Jiang, J. and Hu, P., 2013. Simultaneous determination of olmesartan and amlodipine in human plasma and urine by ultra-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography B*, *938*, pp.27-34.

Qin, F., Wang, D., Yang, S., Jing, L., Xiong, Z. and Li, F., 2011. Quantitative determination of lisinopril in human plasma by high performance liquid chromatography–tandem mass spectrometry and its application in a pharmacokinetic study. *Biomedical Chromatography*, *26*(6), pp.691-696.

Qin, W., Zhang, Z., Tian, Y., Xu, F., Wang, N. and Chen, Y., 2007. Rapid quantification of lisinopril in human plasma by liquid chromatography/tandem mass spectrometry. *Biomedical Chromatography*, *21*(4), pp.415-421.

Qu, Y., Brady, K., Apilado, R., O'Malley, T., Reddy, S., Chitkara, P., Ibarra, C., Alexander, R.V. and Dervieux, T., 2017. Capillary blood collected on volumetric absorptive microsampling (VAMS) device for monitoring hydroxychloroquine in rheumatoid arthritis patients. *Journal of Pharmaceutical and Biomedical Analysis*, *140,* pp.334-341.

Quiroga, P.A., Yuln, G.V., Palummo, M., Cingolani, A., Dall, L.L. and Volonté, M.G., 2001. Comparative bioavailability of diltiazem in prolonged-release oral preparations. *Drug Development and Industrial Pharmacy*, *27*(10), pp.1099-1106.

Quraishi, R., Jain, R. and Ambekar, A., 2013. The use of dried blood spot samples in screening drugs of abuse. *Pharmacology & Pharmacy*, *4*(02), pp.152-159.

Rago, B. and Negahban, A., 2016. Outsource-ability of high-resolution MS-based DMPK assays. *Bioanalysis*, 8(16), pp.1641-1644.

Ramakrishnan, P., Nair, S. and Rangiah, K., 2016. A method for comparative metabolomics in urine using high resolution mass spectrometry. *Journal of Chromatography A*, *1443*, pp.83-92.

Ramanathan, R. and Korfmacher, W., 2012. The emergence of high-resolution MS as the premier analytical tool in the pharmaceutical bioanalysis arena. *Bioanalysis, 4*(5), pp.467-469.

Ramanathan, R., Jemal, M., Ramagiri, S., Xia, Y.Q., Humpreys, W.G., Olah, T. and Korfmacher, W.A., 2011. It is time for a paradigm shift in drug discovery bioanalysis: from SRM to HRMS. *Journal of Mass Spectrometry*, *46*(6), pp.595-601.

Ramani, A.V., Sengupta, P. and Mullangi, R., 2009. Development and validation of a highly sensitive and robust LC-ESI-MS/MS method for simultaneous quantitation of simvastatin acid, amlodipine and valsartan in human plasma: application to a clinical pharmacokinetic study. *Biomedical Chromatography*, *23*(6), pp.615-622.

Rao, R.N., Bompelli, S. and Maurya, P.K., 2011. High-performance liquid chromatographic determination of anti-hypertensive drugs on dried blood spots using a fluorescence detector–method development and validation. *Biomedical Chromatography*, *25*(11), pp.1252-1259.

Rao, R.N., Raju, S.S., Vali, R.M. and Sankar, G.G., 2012. Liquid chromatography–mass spectrometric determination of losartan and its active metabolite on dried blood spots. *Journal of Chromatography B*, *902*, pp.47-54.

Rapoff, M.A., 2010. *Adherence to paediatric medical regimens*. Springer Science, New York, pp.232.

Ravi, V.B., Mullangi, R., Inamadugu, J.K., Pilli, N.R., Gajula, R. and Ponneri, V., 2012. Simultaneous determination of atorvastatin and niacin in human plasma by LC-MS/MS and its application to a human pharmacokinetic study. *Biomedical Chromatography*, *26*(11), pp.1436-1443.

Reinoso, R.F., Sanchez Navarro, A., Garcia, M.J. and Prous, J.R., 2002. Preclinical pharmacokinetics of statins. *Methods and Findings, Experimental Clinical Pharmacology*, *24*(9), pp.593-613.

Rezk, M.R. and Badr, K.A., 2014. Development, optimisation and validation of a highly sensitive UPLC–ESI-MS/MS method for simultaneous quantification of amlodipine, benazepril and benazeprilat in human plasma: Application to a bioequivalence study. *Journal of Pharmaceutical and Biomedical Analysis*, *98*, pp.1-8.

Rincón, J.P. and Meesters, R.J., 2014. Evaluation of peripheral blood microsampling techniques in combination with liquid chromatography-high resolution mass spectrometry for the determination of drug pharmacokinetics in clinical studies. *Drug Testing and Analysis*, *6*(6), pp.568-577.

Roberts, M.E., Wheeler, K.J. and Neiheisel, M.B., 2014. Medication adherence part three: Strategies for improving adherence. *Journal of the American Association of Nurse Practitioners*, *26*(5), pp.281-287.

Robertson, J., Shilkofski, N., 2005. *The Harriet Lane Handbook*. 17th edition. pp.557.

Rochat, B., Kottelat, E. and McMullen, J., 2012. The future key role of LC–high-resolution-MS analyses in clinical laboratories: a focus on quantification. *Bioanalysis, 4*(24), pp.2939-2958.

Roehr, B., 2013. Potential benefits of" well engaged patients" are akin to those from a blockbuster drug, say experts. *BMJ: British Medical Journal (Online)*, *346*.

Rogatsky, E. and Stein, D., 2005. Evaluation of matrix effect and chromatography efficiency: new parameters for validation of method development. *Journal of the American Society for Mass Spectrometry*, *16*(11), pp.1757-1759.

Ruddy, K., Mayer, E. and Partridge, A., 2009. Patient adherence and persistence with oral anticancer treatment. *CA: A Cancer Journal for Clinicians*, *59*(1), pp.56-66.

Ruppar, T.M., Dobbels, F., Lewek, P., Matyjaszczyk, M., Siebens, K. and De Geest, S.M., 2015. Systematic review of clinical practice guidelines for the improvement of medication adherence. *International Journal of Behavioral Medicine*, *22*(6), pp.699-708.

Ruppar, T.M., Dunbar-Jacob, J.M., Mehr, D.R., Lewis, L. and Conn, V.S., 2017. Medication adherence interventions among hypertensive black adults: a systematic review and meta-analysis. *Journal of Hypertension*, *35*(6), pp.1145-1154.

Sabate E, Adherence to Long Term Therapies: Evidence for Action, World Health Organisation, 2003. http://apps.who.int/medicinedocs/en/d/Js4883e/6.html (assessed 18.10.2014).

Salvadori, M.C., Moreira, R.F., Borges, B.C., Andraus, M.H., Azevedo, C.P., Moreno, R.A. and Borges, N.C., 2009. Simultaneous determination of losartan and hydrochlorothiazide in human plasma by LC/MS/MS with electrospray ionisation and its application to pharmacokinetics. *Clinical and Experimental Hypertension*, *31*(5), pp.415-427.

Sapkota, S., Brien, J.A., Greenfield, J. and Aslani, P., 2015. A systematic review of interventions addressing adherence to anti-diabetic medications in patients with type 2 diabetes—impact on adherence. *PloS One*, *10*(2), pp.e0118296.

Sargent, M., 2013. *Guide to achieving reliable quantitative LC-MS measurements*. RSC Analytical Methods Committee, Teddington, Middlesex, United Kingdom, pp.2-61.

Schoenthaler, A., Knafl, G.J., Fiscella, K. and Ogedegbe, G., 2017. Addressing the social needs of hypertensive patients: the role of patient–provider communication as a predictor of medication adherence. *Circulation: Cardiovascular Quality and Outcomes*, *10*(9), p.e003659.

Schultz, G. and Henion, J., 2017. Fundamentals of LC-MS/MS for Regulated Bioanalysis. In *Regulated Bioanalysis: Fundamentals and Practice,* Springer International Publishing, pp.103-120.

Seabury, S.A., Gupta, C.N., Philipson, T.J., Henkhaus, L.E. and PhRMA Medication Adherence Advisory Council, 2014. Understanding and overcoming barriers to medication adherence: a review of research priorities. *Journal of Managed Care Pharmacy*, *20*(8), pp.775-783.

Selinger, K., Fung, E.N. and Bryan, P., 2014. Chapter 16: *Bioanalytical method validation and bioanalysis in regulated settings*. Specification of Drug Substances and Products, Riley, CM; Rosanske, TW; Riley, SRR, Eds. Elsevier: Oxford, pp.325-363.

Selvan, P.S. and Pal, T.K., 2009. Chromatography–tandem mass spectrometry method for the simultaneous quantitation of metoprolol succinate and simvastatin in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, *49*(3), pp.780-785.

Selvan, P.S., Gowda, K.V., Mandal, U., Solomon, W.S. and Pal, T.K., 2007. Simultaneous determination of fixed dose combination of nebivolol and valsartan in human plasma by liquid chromatographic-tandem mass spectrometry and its application to pharmacokinetic study. *Journal of Chromatography B*, *858*(1), pp.143-150.

Shah, H.J., Kataria, N.B., Subbaiah, G. and Patel, C.N., 2009. Simultaneous LC–MS–MS analysis of valsartan and hydrochlorothiazide in human plasma. *Chromatographia*, *69*(9-10), pp.1055-1060.

Shah, J.V., Parekh, J.M., Shah, P.A., Shah, P.V., Sanyal, M. and Shrivastav, P.S., 2017. Application of an LC-MS/MS method for the analysis of amlodipine, valsartan and hydrochlorothiazide in polypill for a bioequivalence study. *Journal of Pharmaceutical Analysis*, *7*, pp.309-316.

Shah, J.V., Shah, P.A., Shah, P.V., Sanyal, M. and Shrivastav, P.S., 2016. Fast and sensitive LC–MS/MS method for the simultaneous determination of lisinopril and hydrochlorothiazide in human plasma. *Journal of Pharmaceutical Analysis*, *7*(3), pp.163-169.

Shah, N.M., Hawwa, A.F., Millership, J.S., Collier, P.S. and McElnay, J.C., 2013. A simple bioanalytical method for the quantification of antiepileptic drugs in dried blood spots. *Journal of Chromatography B*, *923*, pp.65-73.

Shah, N.M., Hawwa, A.F., Millership, J.S., Collier, P.S., Ho, P., Tan, M.L., Peake, D., Tirupathi, S., Bothwell, J., Bailie, N. and Shepherd, C., 2013. Adherence to antiepileptic medicines in children: A multiple-methods assessment involving dried blood spot sampling. *Epilepsia*, *54*(6), pp.1020-1027.

Sharma, A., Jaiswal, S., Shukla, M. and Lal, J., 2014. Dried blood spots: concepts, present status, and future perspectives in bioanalysis. *Drug Testing and Analysis*, *6*(5), pp.399-414.

Shen, Y., Zhang, Y.F., Chen, X.Y., Guo, L.X. and Zhong, D.F., 2012. Pharmacokinetics and bioequivalence of atorvastatin calcium tablets in healthy male Chinese volunteers. *Zhonghua Xin Xue Guan Bing Za Zhi*, *40*(3), pp.243-247.

Shentu, J., Fu, L., Zhou, H., Hu, X.J., Liu, J., Chen, J. and Wu, G., 2012. Determination of amlodipine in human plasma using automated online solid-phase extraction HPLC–tandem mass spectrometry: Application to a bioequivalence study of Chinese volunteers. *Journal of Pharmaceutical and Biomedical Analysis*, *70*, pp.614-618.

Shipkova, M. and Svinarov, D., 2016. LC–MS/MS as a tool for TDM services: Where are we? *Clinical Biochemistry*, *49*(13), pp.1009-1023.

Shipkova, M., Rapp, S., Rigo-Bonnin, R., Wieland, E. and Peter, A., 2017. Therapeutic Drug Monitoring of Everolimus: Comparability of Concentrations Determined by 2 Immunoassays and a Liquid Chromatography Tandem Mass Spectrometry Method. *Therapeutic drug monitoring*, *39*(2), pp.102-108.

Sica, D.A., Gehr, T.W. and Ghosh, S., 2005. Clinical pharmacokinetics of losartan. *Clinical Pharmacokinetics*, *44*(8), pp.797-814.

Silva, S.C.R., Rezende, G.R.D. and Boralli, V.B., 2014. Quick and simple LC-MS/MS method for the determination of simvastatin in human plasma: application to pharmacokinetics and bioequivalence studies. *Brazilian Journal of Pharmaceutical Sciences*, *50*(3), pp.543-550.

Simões, S.S., Ajenjo, A.C. and Dias, M.J., 2017. Dried blood spots combined to an UPLC–MS/MS method for the simultaneous determination of drugs of abuse in forensic toxicology. *Journal of Pharmaceutical and Biomedical Analysis*. 147, pp.634-644.

Singh, P., 2017. Medication adherence: Adjunct to patient outcomes. *Journal of the Practice of Cardiovascular Sciences*, *3*(1), p.8.

Singleton, C., 2012. Recent advances in bioanalytical sample preparation for LC–MS analysis. *Bioanalysis, 4*(9), pp.1123-1140.

Snyder, L.R., Kirkland, J.J. and Glajch, J.L., 2012. *Practical HPLC Method Development*. John Wiley & Sons, pp.145-149.

Spielberg, F., Critchlow, C., Vittinghoff, E., Coletti, A.S., Sheppard, H., Mayer, K.H., Metzger, D., Judson, F.N., Buchbinder, S., Chesney, M. and Gross, M., 2000. Home collection for frequent HIV testing: acceptability of oral fluids, dried blood spots and telephone results. *Aids*, *14*(12), pp.1819-1828.

Spooner, N., 2013. A dried blood spot update: still an important bioanalytical technique? *Bioanalysis*, *5*(8), pp.879-883.

Spooner, N., Denniff, P., Michielsen, L., De Vries, R., Ji, Q.C., Arnold, M.E., Woods, K., Woolf, E.J., Xu, Y., Boutet, V. and Zane, P., 2015. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. *Bioanalysis, 7,* pp.653–659.

Sridharan, D., Thenmozhi, A. and Sundaranandavalli, S., 2010. Bioanalytical method development and validation of atenolol in human plasma by LC-MS. *Asian Journal of Pharmacy and Clinical Research*, *3*, pp.92-94.

Srinivas, N.R., 2016. Should commonly prescribed drugs be avoided as internal standard choices in new assays for clinical samples? *Bioanalysis,* pp.607-610

Sripalakit, P., Nermhom, P. and Saraphanchotiwitthaya, A., 2006. Validation and pharmacokinetic application of a method for determination of doxazosin in human plasma by high-performance liquid chromatography. *Biomedical Chromatography*, *20*(8), pp.729-735.

Sripalakit, P., Nermhom, P., Maphanta, S., Polnok, S., Jianmongkol, P. and Saraphanchotiwitthaya, A., 2005. Bioequivalence evaluation of two formulations of doxazosin tablet in healthy Thai male volunteers. *Drug Development and Industrial Pharmacy*, *31*(10), pp.1035-1040.

Stegemann, S., Ecker, F., Maio, M., Kraahs, P., Wohlfart, R., Breitkreutz, J., Zimmer, A., Bar-Shalom, D., Hettrich, P. and Broegmann, B., 2010. Geriatric drug therapy: neglecting the inevitable majority. *Ageing Research Reviews*, *9*(4), pp.384-398.

Stekler, J.D., Ure, G., Dragavon, J., Chang, M. and Coombs, R.W., 2017. Detection of HIV RNA in dried blood spots and oral fluids. *AIDS*, *31*(8), pp.1191-1193.

Stevenson, D., 2011. Bioanalysis through the ages. *Bioanalysis*, *3*(24), pp.2681-2683.

Stove, C., 2015. *New sampling strategies in toxicology and therapeutic drug monitoring*. Future Science, pp.121 - 132.

Sturm, R., Henion, J., Abbott, R. and Wang, P., 2015. Novel membrane devices and their potential utility in blood sample collection prior to analysis of dried plasma spots. *Bioanalysis. 7*(16), pp.1987-2002.

Sturm, R.M., Jones, B.R., Mulvana, D.E. and Lowes, S., 2016. HRMS using a Q-Exactive series mass spectrometer for regulated quantitative bioanalysis: how, when, and why to implement. *Bioanalysis*, *8*(16), pp.1709-1721.

Suyagh, M.F., Iheagwaram, G., Kole, P.L., Millership, J., Collier, P., Halliday, H. and McElnay, J.C., 2010. Development and validation of a dried blood spot–HPLC assay for the determination of metronidazole in neonatal whole blood samples. *Analytical and Bioanalytical Chemistry*, *397*(2), pp.687-693.

Takyi-Williams, J., Liu, C.F. and Tang, K., 2015. Ambient ionisation MS for bioanalysis: recent developments and challenges. *Bioanalysis, 7*(15), pp.1901-1926.

Tan, A., Jin, W., Deng, F., Hussain, S., Musuku, A. and Massé, R., 2009. Bioanalytical method development and validation using incurred samples—Simultaneous quantitation of ramipril and ramiprilat in human EDTA plasma by LC–MS/MS. *Journal of Chromatography B*, *877*(29), pp.3673-3680.

Taneja, I., Erukala, M., Raju, K.S.R. and Singh, S.P., 2013. Dried blood spots in bioanalysis of antimalarials: relevance and challenges in quantitative assessment of antimalarial drugs. *Bioanalysis*, *5*(17), pp.2171-2186.

Tang, L. and Kebarle, P., 1993. Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution. *Analytical Chemistry*, *65*(24), pp.3654-3668.

Tanna, S. and Lawson, G., 2011. Analytical methods used in conjunction with dried blood spots. *Analytical Methods*, *3*(8), pp.1709-1718.

Tanna, S. and Lawson, G., 2014. Cardiovascular drug medication adherence assessed by dried blood spot analysis. *Journal of Analytical and Bioanalytical Techniques*, (S12), pp.001-003.

Tanna, S. and Lawson, G., 2014. Dried blood spot analysis to assess medication adherence and to inform personalization of treatment. *Bioanalysis*, *6*(21), pp.2825-2838.

Tanna, S. and Lawson, G., 2015. Editorial - Self-sampling and quantitative analysis of DBS: can it shift the balance in over-burdened healthcare systems? *Bioanalysis*, *7*(16), pp.1963-1966.

Tanna, S. and Lawson, G., 2016. *Analytical Chemistry for Assessing Medication Adherence*. 1st Edition, Elsevier, pp.51-59.

Tanna, S., Bernieh, D. and Lawson, G., 2015. LC-HRMS analysis of dried blood spot samples for assessing adherence to cardiovascular medications. *Journal of Bioanalysis and Biomedicine, 7*, pp.001-005.

Tessier, A., Boyer, L., Husky, M., Baylé, F., Llorca, P.M. and Misdrahi, D., 2017. Medication adherence in schizophrenia: The role of insight, therapeutic alliance and perceived trauma associated with psychiatric care. *Psychiatry Research*, *257*, pp.315-321.

Timmerman, P., White, S., Globig, S., Lüdtke, S., Brunet, L. and Smeraglia, J., 2011. EBF recommendation on the validation of bioanalytical methods for dried blood spots. *Bioanalysis, 3*(14), pp.1567-1575.

Tippabhotla, S.K., Betha, M.R., Gadiko, C., Battula, R., Nakkawar, M., Cheerla, R., Khan, S.M., Yergude, S., Thota, S. and Vobalaboina, V., 2013. Bioequivalence of Fixed Dose Combination of Atorvastatin 10 mg and Aspirin 150 mg Capsules: A Randomized, Open-Label, Single-Dose, Two-way Crossover Study in Healthy Human Subjects. *Drug Research*, *63*(05), pp.250-257.

Tomaszewski, M., White, C., Patel, P., Masca, N., Damani, R., Hepworth, J., Samani, N.J., Gupta, P., Madira, W., Stanley, A. and Williams, B., 2014. High rates of non-adherence to antihypertensive treatment revealed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) urine analysis. *Heart*, *100*(11), pp.855-861.

Tomková, J., Ondra, P., Kocianová, E. and Václavík, J., 2017. Fast and sensitive analysis of beta blockers by ultra-high-performance liquid chromatography coupled with ultra-high-resolution TOF mass spectrometry. *Biomedical Chromatography*, *31*(7), pp1-16.

Tomtec pre-cut dry media spot slide, 2015. http://www.tomtec.com/dried-blood-spots.html, (accessed 02.09.17).

Trajan Scientific and Medical, Hemapen microsampling device, 2015. https://www.trajanscimed.com/pages/hemapen, (accessed 09.09.17).

Trajan Scientific and Medical, precision biosampling, hemaPen, 2016. https://www.trajanscimed.com/pages/hemapen (accessed on 10.06.17).

Tretzel, L., Thomas, A., Piper, T., Hedeland, M., Geyer, H., Schänzer, W. and Thevis, M., 2016. Fully automated determination of nicotine and its major metabolites in whole blood by means of a DBS online-SPE LC-HR-MS/MS approach for sports drug testing. *Journal of Pharmaceutical and Biomedical Analysis*, *123*, pp.132-140.

Tsakalof, A., Bairachtari, K. and Georgarakis, M., 2002. Development of a liquid chromatography–mass spectrometry method for monitoring the angiotensin-converting enzyme inhibitor lisinopril in serum. *Journal of Chromatography B*, *783*(2), pp.425-432.

Tutunji, M.F., Ibrahim, H.M., Khabbas, M.H. and Tutunji, L.F., 2009. Simultaneous determination of bisoprolol and hydrochlorothiazide in human plasma by HPLC coupled with tandem mass spectrometry. *Journal of Chromatography B*, *877*(16-17), pp.1689-1697.

UNICEF, 2009. Taking blood from infants for the HIV DNA PCR test - Standard Operating Procedures. National Health Laboratory Services, UNICEF, De Beers Fund, pp. 25.

Van Dalem, J., Krass, I. and Aslani, P., 2012. Interventions promoting adherence to cardiovascular medicines. *International Journal of Clinical Pharmacy*, *34*(2), pp.295-311.

Van Griensven, J.M.T., Schoemaker, R.C., Cohen, A.F., Luus, H.G., Seibert-Grafe, M. and Röthig, H.J., 1995. Pharmacokinetics, pharmacodynamics and bioavailability of the ACE inhibitor ramipril. *European Journal of Clinical Pharmacology*, *47*(6), pp.513-518.

Veenhof, H., Koster, R.A., Alffenaar, J.W.C., Berger, S.P., Bakker, S.J. and Touw, D.J., 2017. Clinical Validation of Simultaneous Analysis of Tacrolimus, Cyclosporine A, and Creatinine in Dried Blood Spots in Kidney Transplant Patients. *Transplantation*, *101*(7), pp.1727-1733.

Velligan, D.I., Weiden, P.J., Sajatovic, M., Scott, J., Carpenter, D., Ross, R. and Docherty, J.P., 2009. The expert consensus guideline series: adherence problems in patients with serious and persistent mental illness. *The Journal of Clinical Psychiatry*, *70*(4), pp.1-48.

Vergin, H. and Nitsche, V., 1989. Oral bioavailability of atenolol. *Journal of International Medical Research*, *17*(5), pp.417-425.

Verhaeghe, T., Dillen, L., Stieltjes, H., Zwart, L.D., Feyen, B., Diels, L., Vroman, A. and Timmerman, P., 2017. The application of capillary microsampling in GLP toxicology studies. *Bioanalysis*, *9*(7), pp.531-540.

Verougstraete, N., Lapauw, B., Van Aken, S., Delanghe, J., Stove, C. and Stove, V., 2017. Volumetric absorptive microsampling at home as an alternative tool for the monitoring

of HbA1c in diabetes patients. *Clinical Chemistry and Laboratory Medicine (CCLM)*, *55*(3), pp.462-469.

Verplaetse, R. and Henion, J., 2016. Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS. *Drug Testing and Analysis*, *8*(1), pp.30-38.

Vetrova, E.V., Lekar, A.V., Filonova, O.V., Borisenko, S.N., Maksimenko, E.V. and Borisenko, N.I., 2015. Study of Simvastatin Self-Association Using Electrospray-Ionisation Mass Spectrometry. *Journal of Applied Spectroscopy*, *82*(3), pp.337-342.

Vlase, L., Popa, D.S., Muntean, D. and Achim, M., 2010. A New LC/MS/MS Method for Determination of Lisinopril in Human Plasma. *Studia Universitatis Babeş-Bolyai Chemia*, *55*(1), pp.91-101.

Volpe, M., Chin, D. and Paneni, F., 2010. The challenge of polypharmacy in cardiovascular medicine. *Fundamental & Clinical Pharmacology*, *24*(1), pp.9-17.

Vu, D.H., Koster, R.A., Bolhuis, M.S., Greijdanus, B., Altena, R.V., Nguyen, D.H., Brouwers, J.R.B.J., Uges, D.R.A. and Alffenaar, J.W.C., 2014. Simultaneous determination of rifampicin, clarithromycin and their metabolites in dried blood spots using LC–MS/MS. *Talanta*, *121*, pp.9-17.

Wagner, G.J. and Rabkin, J.G., 2000. Measuring medication adherence: are missed doses reported more accurately than perfect adherence?. *AIDS Care*, *12*(4), pp.405-408.

Wagner, M., Tonoli, D., Varesio, E. and Hopfgartner, G., 2014. The use of mass spectrometry to analyze dried blood spots. *Mass Spectrometry Reviews*, *35*(3), pp.361-438.

Walker HK, Hall WD, and Hurst JW, Clinical Methods, *The History, Physical and Laboratory examinations*, Boston, Butterworths 1990, Chapter 151, p 718-719.

Wang, J., Luzum, J.A., Phelps, M.A. and Kitzmiller, J.P., 2015. Liquid chromatography–tandem mass spectrometry assay for the simultaneous quantification of simvastatin,

lovastatin, atorvastatin, and their major metabolites in human plasma. *Journal of Chromatography B*, *983*, pp.18-25.

Waterman, K.C., Adami, R.C. 2005. Accelerated aging: Prediction of chemical stability of pharmaceuticals. *International Journal of Pharmacy, 293,* pp.101–125

Wayne PA. Blood Collection on Filter Paper for Newborn Screening Programs; Approved Standard— Clin Lab Stand Inst. NBS01-A6: (2013) Sixth Edition, pp.32-39.

Weber, J., Oberfeld, S., Bonse, A., Telger, K., Lingg, R. and Hempel, G., 2017. Validation of a dried blood spot method for therapeutic drug monitoring of citalopram, mirtazapine and risperidone and its active metabolite 9-hydroxyrisperidone using HPLC–MS. *Journal of Pharmaceutical and Biomedical Analysis*, *140*, pp.347-354.

Wheeler, K.J., Roberts, M.E. and Neiheisel, M.B., 2014. Medication adherence part two: predictors of nonadherence and adherence. *Journal of the American Association of Nurse Practitioners*, *26*(4), pp.225-232.

WHO, 2005. HIV rapid test training package: participant manual Blood Collection and handling - DBS: World Health Organisation, pp. 13. http://apps.who.int/iris/bitstream/10665/43315/1/9241593563_eng.pdf, (accessed 15.06.16)

Wilhelm, A.J., den Burger, J.C. and Swart, E.L., 2014. Therapeutic drug monitoring by dried blood spot: progress to date and future directions. *Clinical Pharmacokinetics*, *53*(11), pp.961-973.

Wolff, K., 2017. *Detection of Drug Misuse: Biomarkers, Analytical Advances and Interpretation*. Royal Society of Chemistry, London, pp.127-146.

Wong, P., Pham, R., Bruenner, B.A. and James, C.A., 2010. Increasing efficiency for dried blood spot analysis: prospects for automation and simplified sample analysis. *Bioanalysis*, *2*(11), pp.1787-1789.

World Health Organization and Centers for Disease Control and Prevention, 2005. Guidelines for assuring the accuracy and reliability of HIV rapid testing: applying a quality system approach. World Health Organisation, pp.25.

World Health Organization, Cardiovascular diseases (CVDs). http://www.who.int/mediacentre/factsheets/fs317/en/, 2016 (accessed 13.07.16).

Wu, F.L.L., Chen, P.F., Lee, Y.J. and Chen, R.R.L., 2003. Comparative pharmacokinetics of two atenolol products. *Journal of Food and Drug Analysis*, *11*(1), pp.4-7.

Xian, F., Hendrickson, C.L. and Marshall, A.G., 2012. High resolution mass spectrometry. *Analytical chemistry*, *84*(2), pp.708-719.

Xie, C., Zhong, D., Yu, K. and Chen, X., 2012. Recent advances in metabolite identification and quantitative bioanalysis by LC–Q-TOF MS. *Bioanalysis*, *4*(8), pp.937-959.

Xu, Q.A. and Madden, T.L. eds., 2012. *LC-MS in drug bioanalysis*. Springer Science and Business Media, pp33-38.

Xue, K.S., Cai, W., Tang, L. and Wang, J.S., 2016. Aflatoxin B 1-lysine adduct in dried blood spot samples of animals and humans. *Food and Chemical Toxicology*, *98*, pp.210-219.

Yacoub, M., Alawi, M. and Arafat, T., 2013. Simultaneous determination of amlodipine and atorvastatin with its metabolites; ortho and para hydroxy atorvastatin; in human plasma by LC–MS/MS. *Journal of Chromatography B*, *917*, pp.36-47.

Yang, D.J. and Hwang, L.S., 2006. Study on the conversion of three natural statins from lactone forms to their corresponding hydroxy acid forms and their determination in Pu-Erh tea. *Journal of Chromatography A*, *1119*(1), pp.277-284.

Yang, H., Feng, Y. and Luan, Y., 2003. Determination of Simvastatin in human plasma by liquid chromatography–mass spectrometry. *Journal of Chromatography B*, *785*(2), pp.369-375.

Yang, L., Guo, T., Xia, D.Y. and Zhao, L.S., 2012. Pharmacokinetics of losartan and its active carboxylic acid metabolite E-3174 in five ethnic populations of China. *Journal of Clinical Pharmacy and Therapeutics*, *37*(2), pp.226-231.

Yang, Q., Chang, A., Ritchey, M.D. and Loustalot, F., 2017. Antihypertensive medication adherence and risk of cardiovascular disease among older adults: A population-based cohort study. *Journal of the American Heart Association*, *6*(6), p.e006056.

Ye, Z. and Gao, H., 2017. Evaluation of sample extraction methods for minimizing hematocrit effect on whole blood analysis with volumetric absorptive microsampling. *Bioanalysis. 9*(4), pp.2015 – 2024.

Youhnovski, N., Bergeron, A., Furtado, M. and Garofolo, F., 2011. Pre-cut dried blood spot (PCDBS): an alternative to dried blood spot (DBS) technique to overcome hematocrit impact. *Rapid Communications in Mass Spectrometry*, *25*(19), pp.2951-2958.

Yu, Q., Hu, Z.Y., Zhu, F.Y., Zhu, J.H., Wan, L.L., Li, Y. and Guo, C., 2011. HPLC–MS–MS for the simultaneous determination of atorvastatin and amlodipine in plasma of hypertensive patients. *Chromatographia*, *73*(3-4), pp.257-262.

Yuan, B., Wang, X., Zhang, F., Jia, J. and Tang, F., 2008. Simultaneous determination of ramipril and its active metabolite ramiprilat in human plasma by LC–MS–MS. *Chromatographia*, *68*(7-8), pp.533-539.

Zakaria, R., Allen, K.J., Koplin, J.J., Roche, P. and Greaves, R.F., 2016. Advantages and challenges of dried blood spot analysis by mass spectrometry across the total testing process. *EJIFCC*, *27*(4), pp.288 - 317.

Zakeri-Milani, P., Valizadeh, H., Islambulchilar, Z. and Nemati, M., 2010. Pharmacokinetic and bioequivalence study of two brands of valsartan tablets in healthy male volunteers. *Arzneimittelforschung-Drug Research*, *60*(2), pp.76-80.

Zane, P. and Emmons, G.T., 2013. *Microsampling in pharmaceutical bioanalysis*. Future Science Limited, London, pp.3-97.

Zeller, A., Taegtmeyer, A., Martina, B., Battegay, E. and Tschudi, P., 2008. Physicians' ability to predict patients' adherence to antihypertensive medication in primary care. *Hypertension Research*, *31*(9), pp.1765-1771.

Zeng, A., Wang, C., Yuan, B., Yang, G. and Fu, Q., 2010. The influence of chirality, physicochemical properties, and permeation enhancers on the transdermal permeation of amlodipine across rat skin. *Drug Development and Industrial Pharmacy*, *36*(6), pp.724-734.

Zhang, N., Yang, A., Rogers, J.D. and Zhao, J.J., 2004. Quantitative analysis of simvastatin and its β-hydroxy acid in human plasma using automated liquid–liquid extraction based on 96-well plate format and liquid chromatography-tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, *34*(1), pp.175-187.

Zhang, N.R., Yu, S., Tiller, P., Yeh, S., Mahan, E. and Emary, W.B., 2009. Quantitation of small molecules using high-resolution accurate mass spectrometers–a different approach for analysis of biological samples. *Rapid Communications in Mass Spectrometry*, *23*(7), pp.1085-1094.

Zheng, J.H., Guida, L.A., Rower, C., Castillo-Mancilla, J., Meditz, A., Klein, B., Kerr, B.J., Langness, J., Bushman, L., Kiser, J. and Anderson, P.L., 2014. Quantitation of tenofovir and emtricitabine in dried blood spots (DBS) with LC–MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, *88*, pp.144-151.

Zhi, J., Moore, R., Kanitra, L. and Mulligan, T.E., 2003. Effects of orlistat, a lipase inhibitor, on the pharmacokinetics of three highly lipophilic drugs (amiodarone, fluoxetine, and simvastatin) in healthy volunteers. *The Journal of Clinical Pharmacology*, *43*(4), pp.428-435.

Zhou, N., Liang, Y.Z., Chen, B.M., Wang, P., Chen, X. and Liu, F.P., 2008. Development and Validation of LC—MS Method for the Determination of Lisinopril in Human Plasma and its Application in a Bioequivalence Study. *Journal of Chromatographic Science*, *46*(10), pp.848-853.

Zhu, Z., Vachareau, A. and Neirinck, L., 2002. Liquid chromatography–mass spectrometry method for determination of ramipril and its active metabolite ramiprilat in human plasma. *Journal of Chromatography B*, *779*(2), pp.297-306.

Zullig, L.L., Ramos, K. and Bosworth, H.B., 2017. Improving Medication Adherence in Coronary Heart Disease. *Current Cardiology Reports*, *19*(11), pp.113-126.

Appendix 1 Standard operating procedure for the preparation of calibration and validation standards for the 11 target cardiovascular drugs and internal standard in whole blood

1. Introduction

The purpose of this document is to describe the protocol to use in the preparation of calibration and validation standards for amlodipine besylate salt, atenolol, atorvastatin calcium salt, bisoprolol hemifumarate salt, diltiazem hydrochloride, doxazosin mesylate salt, lisinopril, losartan potassium salt, ramipril, simvastatin, valsartan and atenolol D_7 (Internal standard), in human whole blood.

Table A1.1 Proposed calibration ranges of the 11 target cardiovascular drugs in human whole blood

Drug	Proposed calibration ranges (ng/ml)
Amlodipine	0.5, 1, 5, 10, 25, 50, 100
Atenolol	10, 20, 50, 100, 200, 500, 1000, 1500
Atorvastatin	0.5, 1, 5, 10, 25, 50, 100
Bisoprolol	0.1, 0.5, 1, 5, 10, 25, 50, 100
Diltiazem	0.5, 1, 5, 10, 50, 100, 300, 600
Doxazosin	0.1, 0.5, 1, 5, 10, 25, 50, 100
Lisinopril	0.1, 0.5, 1, 5, 10, 25, 50, 100
Losartan	5, 10, 25, 50, 100, 250, 500, 1000
Ramipril	0.1, 0.5, 1, 5, 10, 25, 50, 100
Simvastatin	0.1, 0.5, 1, 5, 10, 25, 50, 100
Valsartan	50, 100, 250, 500, 1000, 2000, 3000, 4000

2. Safety

For the preparation and handling of the cardiovascular drugs, Personal Protective Equipment (PPE) must be worn as per the appropriate risk assessment and completed COSHH forms of each target drug. For the preparation of blood samples, a laboratory coat and disposable latex gloves should be worn. Hands should be washed and disinfected before and after handling blood samples. Care should be taken to cover any cuts on the hands using waterproof dressings or plasters. If spillage occurs the contaminated area should be cleaned immediately using a suitable disinfectant and disposable paper towels. Benches should be wiped clean with a suitable disinfectant after each work session.

3. Storage of blood samples

Following collection of blood samples, the samples in specimen tubes should be stored in number or code labelled re-sealable polythene bags in the refrigerator in Lab HB 00.15.

4. Disposal

Discarded blood spot sampling paper and any contaminated materials used to clean spillages should be put into the plastic yellow (clinical waste) bag. Discarded specimens in microcentrifuge tubes, specimen tubes and contaminated pipette tips and LC vials should also be put into the plastic yellow (clinical waste) bags. All contaminated sharps e.g. needles should be placed in the rigid walled, yellow and red, sharps bin.

5. Equipment

Analytical balance, volumetric pipettes, eppendorf tubes, volumetric flasks, dried blood spot sampling paper (Specimen collection paper type 903), volumetric absorptive microsampling (VAMS) device.

6. Materials

Amlodipine besylate salt, atenolol, atorvastatin calcium salt, bisoprolol hemifumarate salt, diltiazem hydrochloride, doxazosin mesylate salt, lisinopril, losartan potassium salt, ramipril, simvastatin, valsartan, atenolol D_7 (Internal Standard), methanol (HPLC grade), and HPLC grade water.

7. Diluent preparation (70:30 MeOH: H_2O v/v)

Preparation of 500ml of diluent: Transfer 350ml of methanol in a 500ml measuring cylinder and add HPLC grade water to 500ml. Transfer into a 500ml plain glass bottle and shake the bottle.

8. Standard Stock preparation for the 11 target cardiovascular drugs and internal standard

8.1 Standard stock solution preparation for each target drug

For Amlodipine besylate, atenolol, atorvastatin calcium, bisoprolol hemifumarate, diltiazem hydrochloride, doxazosin mesylate, lisinopril, losartan potassium, ramipril, simvastatin and valsartan prepare a stock solution with concentration 1mg/ml.

Procedure

Weigh 5mg of each target drug directly into a 5ml volumetric flask on an analytical balance. Dissolve with 100% Methanol and make up to the mark, shake well and keep solution refrigerated.

8.1.1 Preparation of intermediate stock solution with concentration 10000ng/ml for each drug

Pipette 100µl of the 1mg/ml standard stock solution of drug into a 10ml volumetric flask and make to the mark with 70:30 MeOH: H_20, v/v. Shake well and keep solution refrigerated

8.2 Preparation of Internal Standard stock solution (Atenolol d_7 10000ng/ml)

Procedure

Weigh 0.5mg of Atenolol d_7 directly into a 50ml volumetric flask on an analytical balance. Dissolve with 100% Methanol and make up to the mark, shake well and keep solution refrigerated.

Dilute stock solution with diluent to produce an extraction solvent with 20ng/ml strength of internal standard.

8.2.1 Preparation of extraction solvent with 20ng/ml concentration of internal standard (Atenolol d_7)

Pipette 20µl of the 10,000ng/ml stock solution of atenolol D_7 into a 10ml volumetric flask and make to the mark with 70:30 MeOH: H_20, v/v. Shake well and keep solution refrigerated.

9. Preparation of multicomponent working solutions for amlodipine, atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan in 5ml and 10ml volumetric flask using (70:30 MeOH: H_2O v/v) from the Standard stock solutions

Table A1.2 – A1.12 shows the API concentrations of each target drug, the volume of stock solution required and the total volume of solution to be prepared.

Table A1.2 Calibration concentrations for amlodipine in solution

	Amlodipine Concentration(ng/ml)	Volume of 10000ng/ml stock required (µL)	Total volume(mL)
Solution 1	1	1	10
Solution 2	5	5	10
Solution 3	10	10	10
Solution 4	50	50	10
Solution 5	100	50	5
Solution 6	250	125	5
Solution 7	500	250	5
Solution 8	1000	500	5

Table A1.3 Calibration concentrations for atenolol in solution

	Atenolol Concentration(ng/ml)	Volume of 1mg/ml stock required (µL)	Total volume(mL)
Solution 1	100	1	10
Solution 2	200	2	10
Solution 3	500	5	10
Solution 4	1000	10	10
Solution 5	2000	10	5
Solution 6	5000	25	5
Solution 7	10000	50	5
Solution 8	15000	75	5

Table A1.4 Calibration concentrations for atorvastatin in solution

	Atorvastatin Concentration(ng/ml)	Volume of 10000ng/ml stock required(µL)	Total volume(mL)
Solution 1	1	1	10
Solution 2	5	5	10
Solution 3	10	10	10
Solution 4	50	50	10
Solution 5	100	50	5
Solution 6	250	125	5
Solution 7	500	250	5
Solution 8	1000	500	5

Table A1.5 Calibration concentrations for bisoprolol in solution

	Bisoprolol Concentration(ng/ml)	Volume of 10000ng/ml stock required (µL)	Total volume(mL)
Solution 1	1	1	10
Solution 2	5	5	10
Solution 3	10	10	10
Solution 4	50	50	10
Solution 5	100	50	5
Solution 6	250	125	5
Solution 7	500	250	5
Solution 8	1000	500	5

Table A1.6 Calibration concentrations for diltiazem in solution

	Diltiazem Concentration(ng/ml)	Volume of 10000ng/ml stock required (µL)	Total volume(mL)
Solution 1	5	5	10
Solution 2	10	10	10
Solution 3	50	50	10
Solution 4	100	100	10
Solution 5	500	250	5
Solution 6	1000	500	5
Solution 7	3000	1500	5
Solution 8	6000	3000	5

Table A1.7 Calibration concentration for doxazosin in solution

	Doxazosin Concentration(ng/ml)	Volume of 10000ng/ml stock required (µL)	Total volume(mL)
Solution 1	1	1	10
Solution 2	5	5	10
Solution 3	10	10	10
Solution 4	50	50	10
Solution 5	100	50	5
Solution 6	250	125	5
Solution 7	500	250	5
Solution 8	1000	500	5

Table A1.8 Calibration concentrations for lisinopril in solution

	Lisinopril Concentration(ng/ml)	Volume of 10000ng/ml stock required (µL)	Total volume(mL)
Solution 1	1	1	10
Solution 2	5	5	10
Solution 3	10	10	10
Solution 4	50	50	10
Solution 5	100	50	5
Solution 6	250	125	5
Solution 7	500	250	5
Solution 8	1000	500	5

Table A1.9 Calibration concentrations for losartan in solution

	Losartan Concentration(ng/ml)	Volume of 10000ng/ml stock required (µL)	Total volume(mL)
Solution 1	50	50	10
Solution 2	100	100	10
Solution 3	250	250	10
Solution 4	500	500	10
Solution 5	1000	500	5
Solution 6	2500	1250	5
Solution 7	5000	2500	5
Solution 8	10000	5000	5

Table A1.10 Calibration concentrations for ramipril in solution

	Ramipril Concentration(ng/ml)	Volume of 10000ng/ml stock required (µL)	Total volume(mL)
Solution 1	1	1	10
Solution 2	5	5	10
Solution 3	10	10	10
Solution 4	50	50	10
Solution 5	100	50	5
Solution 6	250	125	5
Solution 7	500	250	5
Solution 8	1000	500	5

Table A1.11 Calibration concentrations for simvastatin in solution

	Simvastatin Concentration(ng/ml)	Volume of 10000ng/ml stock required (µL)	Total volume(mL)
Solution 1	1	1	10
Solution 2	5	5	10
Solution 3	10	10	10
Solution 4	50	50	10
Solution 5	100	50	5
Solution 6	250	125	5
Solution 7	500	250	5
Solution 8	1000	500	5

Table A1.12 Calibration concentrations for valsartan in solution

	Valsartan Concentration(ng/ml)	Volume of 1mg/ml stock required (µL)	Total volume(mL)
Solution 1	500	5	10
Solution 2	1000	10	10
Solution 3	2500	25	10
Solution 4	5000	50	10
Solution 5	10000	50	5
Solution 6	20000	100	5
Solution 7	30000	150	5
Solution 8	40000	200	5

From Tables A1.2 – A1.12, there will be 8 multicomponent solutions (1, 2, 3, 4, 5, 6, 7, and 8).

10. Preparation of calibration standards in whole blood

For each multicomponent solution (1, 2, 3, 4, 5, 6, 7, and 8) from section 9, pipette 100µL to an eppendorf tube and add 900µL of blood. Mix well by vortexing for 1 minute. This will produce the final calibration concentrations in table 3a – 3k below for each target drug in whole blood. For the blank preparation, pipette 100µl of diluent (extraction solvent containing 10ng/ml internal standard) and add 900µl of blood. Mix well by vortexing for 1 minute.

Table A1.13 – A1.23 show the final calibration concentrations of each target drug in whole blood.

Table A1.13 Final concentrations of amlodipine in whole blood

	Concentration of Amlodipine standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Amlodipine in whole blood (ng/ml)
Standard A	1	100	0.1
Standard B	5	100	0.5
Standard C	10	100	1
Standard D	50	100	5
Standard E	100	100	10
Standard F	250	100	25
Standard G	500	100	50
Standard H	1000	100	100

Table A1.14 Final concentrations of atenolol in whole blood

	Concentration of Atenolol standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Atenolol in whole blood (ng/ml)
Standard A	100	100	10
Standard B	200	100	20
Standard C	500	100	50
Standard D	1000	100	100
Standard E	2000	100	200
Standard F	5000	100	500
Standard G	10000	100	1000
Standard H	15000	100	1500

Table A1.15 Final concentrations of atorvastatin in whole blood

	Concentration of Atorvastatin standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Atorvastatin in whole blood (ng/ml)
Standard A	1	100	0.1
Standard B	5	100	0.5
Standard C	10	100	1
Standard D	50	100	5
Standard E	100	100	10
Standard F	250	100	25
Standard G	500	100	50
Standard H	1000	100	100

Table A1.16 Final concentrations of bisoprolol in whole blood

	Concentration of Bisoprolol standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Bisoprolol in whole blood (ng/ml)
Standard A	1	100	0.1
Standard B	5	100	0.5
Standard C	10	100	1
Standard D	50	100	5
Standard E	100	100	10
Standard F	250	100	25
Standard G	500	100	50
Standard H	1000	100	100

Table A1.17 Final concentrations of diltiazem in whole blood

	Concentration of Diltiazem standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Diltiazem in whole blood (ng/ml)
Standard A	5	100	0.5
Standard B	10	100	1
Standard C	50	100	5
Standard D	100	100	10
Standard E	500	100	50
Standard F	1000	100	100
Standard G	3000	100	300
Standard H	6000	100	600

Table A1.18 Final concentrations of doxazosin in whole blood

	Concentration of Doxazosin standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Doxazosin in whole blood (ng/ml)
Standard A	1	100	0.1
Standard B	5	100	0.5
Standard C	10	100	1
Standard D	50	100	5
Standard E	100	100	10
Standard F	250	100	25
Standard G	500	100	50
Standard H	1000	100	100

Table A1.19 Final concentrations of lisinopril in whole blood

	Concentration of Lisinopril standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Lisinopril in whole blood (ng/ml)
Standard A	1	100	0.1
Standard B	5	100	0.5
Standard C	10	100	1
Standard D	50	100	5
Standard E	100	100	10
Standard F	250	100	25
Standard G	500	100	50
Standard H	1000	100	100

Table A1.20 Final concentrations of losartan in whole blood

	Concentration of Losartan standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Losartan in whole blood (ng/ml)
Standard A	50	100	5
Standard B	100	100	10
Standard C	250	100	25
Standard D	500	100	50
Standard E	1000	100	100
Standard F	2500	100	250
Standard G	5000	100	500
Standard H	10000	100	1000

Table A1.21 Final concentrations of ramipril in whole blood

	Concentration of Ramipril standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Ramipril in whole blood (ng/ml)
Standard A	1	100	0.1
Standard B	5	100	0.5
Standard C	10	100	1
Standard D	50	100	5
Standard E	100	100	10
Standard F	250	100	25
Standard G	500	100	50
Standard H	1000	100	100

Table A1.22 Final concentrations of simvastatin in whole blood

	Concentration of Simvastatin standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Simvastatin in whole blood (ng/ml)
Standard A	1	100	0.1
Standard B	5	100	0.5
Standard C	10	100	1
Standard D	50	100	5
Standard E	100	100	10
Standard F	250	100	25
Standard G	500	100	50
Standard H	1000	100	100

Table A1.23 Final concentrations of valsartan in whole blood

	Concentration of Valsartan standard solution (ng/ml)	Volume of standard to be added to whole blood (µl)	Final Concentration of Valsartan in whole blood (ng/ml)
Standard A	500	100	50
Standard B	1000	100	100
Standard C	2500	100	250
Standard D	5000	100	500
Standard E	10000	100	1000
Standard F	20000	100	2000
Standard G	30000	100	3000
Standard H	40000	100	4000

From Tables A1.13 – A1.23, there will be 8 calibration standards in whole blood (A, B, C, D, E, F, G, H and the blank).

For the purpose of validation studies, Solution C, Solution F and Solution H will be chosen to represent low, medium and high concentrations respectively for the preparation of validation samples.

11. Spotting of blood targets on DBS card

Pipette 30µL of each blood standard (A, B, C, D, E, F, G and H) including blank sample, and spot directly onto the sampling paper using a volumetric pipette. About thirty (30) spots should be made for each blood standard. Allow samples to air dry for at least 2 hours at room temperature. The dried samples should be stored in number or code labelled re-sealable polythene bags in the secure cabinet in Lab HB 00.15. Details of the prepared calibration and validation DBS samples should be logged into the DBS records notebook in the secure cabinet.

12. Spotting of blood targets on VAMS tips

Emmerse the blank VAMS tips at angle of about 45 degrees into each blood standard (A, B, C, D, E, F, G and H) including blank sample. Wait for the tip to go fully red and then count two (2) additional seconds. This ensures that the VAMS substrate accurately samples 10µL of blood. Slowly remove the sampler tip from the microcentrifuge tube and close the clamshell. Allow samples to air dry for at least 2 hours at room temperature. The dried samples should be stored in number or code labelled re-sealable polythene bags in the secure cabinet in Lab HB 00.15. Details of the prepared calibration and validation VAMS samples should be logged into the DBS records notebook in the secure cabinet.

Appendix 2 Participant information leaflet (English version)

PARTICIPANT INFORMATION LEAFLET

Faculty of Health and Life Sciences

Dried blood spot analysis to assess adherence to cardiovascular medications

What is the study?

This project is on the development of a simple non-invasive test to assess adherence to cardiovascular therapy in primary and secondary care.

Cardiovascular disease is one of the biggest killers worldwide affecting 1 in 3 people in the UK. Current care of such patients and increasingly for patients over 50 years old is the prescription of a combination of cardiovascular therapy drugs including beta blockers (BB), ace inhibitors (AI) and statins (ST). There is evidence that up to 60% of patients prescribed cardiovascular drugs do not adhere to their prescribed regimen leading to increases in morbidity, mortality and higher costs of care. The estimated cost of unused prescription medicines in the UK is ~£4 billion annually. A simple test to monitor prescription drug levels would therefore be highly valued.

What will happen?

The programme for this study will involve testing the developed and validated of a dried blood spot (DBS) based analytical method for the principal cardiovascular drugs identified. This analytical method will be used to test DBS samples obtained from participants who are currently taking cardiovascular medication(s) to confirm the successful detection of these drugs in their blood.

How will you be involved?

After reading this Participant Information Leaflet you will be asked to sign a consent form prior to giving a blood spot sample and you will also be asked to complete a small questionnaire. Information requested in this questionnaire will be:

a. Cardiovascular drug(s) prescribed

b. Time since the last dose of the prescribed CVD drug(s) was taken

c. Dose prescribed

The blood spot collection card or device and the questionnaire will remain anonymous.

How is a blood spot sample collected?

The general approach for the collection and use of DBS is as follows: One or two drop(s) of blood are obtained minimally invasively by a simple finger prick or thumb prick procedure. This small volume of blood (~ 25µl) is applied to a sample collection card or other blood sampling device and dried at room temperature for at least 2-3 hours. The sampling can be done almost anywhere. For example, in a laboratory or at home by the participant; in a clinic by a nurse; or in a pharmacy by the community pharmacist. The dried blood spot sample will then be sent to our laboratory for analysis.

How is the blood spot analysed?

In the laboratory, a fixed area of the DBS is extracted, either directly or as a disk punched from the DBS, and the presence of the drug in question is identified by mass spectrometry.

Directions for collection of dried blood sample (DBS) on a Whatman 903 sample collection card

Kit contents:

- DBS Sample collection card (1)
- Alcohol Prep Pad (1)
- Lancet (1)
- Gauze Pad (1)
- Plaster (1)
- Plastic re-sealable bag (1)

1. Fill out the participant reference number on the DBS sample collection card.
2. Warm the skin on a finger or thumb by gentle rubbing.
3. Clean sample site with the alcohol pad provided and allow site to **AIR DRY**.
4. Lance the sample site and wipe away the first blood drop with sterile gauze.
5. Gently apply intermittent pressure near the puncture site to obtain the blood sample on the finger.
6. Allow blood to accumulate on the finger or thumb tip and drop onto the sampling card in the circled area. The blood drop(s) should fall freely to the sampling card.
7. **AVOID TOUCHING** the sampling card and **DO NOT** spread/smear/smudge blood to cover the circled area as this will render the DBS sample invalid.
8. Allow multiple drops to fall on the same circled area until this area is **COMPLETELY** covered and soaked.

9. Once a circled area is covered, start on the next one. At least 2 circles, on the DBS card, must be filled for each sample – this would be from the same finger prick. Over spotting or layering can give rise to erroneous results and will be rejected.
10. Sample cards must then be dried for 2-3 hours at room temperature. Sample cards should be kept apart (i.e. not stacked with each other if there is more than one card) and away from heat.
11. After drying the sample cards must be stored in individual plastic re-sealable bags and are ready for collection or postage to the laboratory with the accompanying completed consent form and adherence questionnaire.

Directions for collection of dried blood sample (DBS) on a VAMS sample collection device

Kit contents:

- Mitra TM (1 clamshell pack containing 4 samplers)
- Alcohol Prep Pad (1)
- Lancet (1)
- Gauze Pad (1)
- Plaster (1)
- Plastic re-sealable bag (1)
- Desiccant (1)

1. Open sealed packaging and remove clamshell package.
2. Label samplers with participant reference number (see Quick Start Guide provided).
3. Uncover the samplers by pulling apart the clamshell and pressing the sides together to create a handle (Quick Start Guide step 2).
4. Clean sample site (side or tip of finger) with the alcohol pad provided and allow site to **AIR DRY**.
5. Lance the sample site and wipe away the first blood drop with sterile gauze.
6. Gently apply intermittent pressure near the puncture site to obtain the blood sample on the finger.
7. Apply sampler tip to surface of blood sample at an angle as shown in Steps 3 and 4 on the Quick Start Guide.
8. Wait for the tip to go fully red and then count 2 additional seconds. Slowly remove the sampler tip from the blood.
9. Repeat 7 and 8 above with the remaining 3 samplers in the 4 – pack.

10. Unfold clamshell to cover sampler tips and press to close.
11. Apply the gauze pad and plaster over the lanced finger.
12. Samplers must then be dried for 2-3 hours at room temperature away from heat or.
13. The covered sampler tips can be immediately placed in the bag with the desiccant.
14. The sampler is now ready for collection or postage to the laboratory with the accompanying completed consent form and mini questionnaire.

Who should I contact if I have further questions?

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CONSENT FORM

Dried blood spot analysis to assess adherence to cardiovascular medications

Participant Reference Number:
(To be completed by research team)

Name of Researchers: Dr S. Tanna, Dr G. Lawson & D. Bernieh

Please initial this box

I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily	☐
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my legal rights being affected.	☐
I understand that the data collected during the study, may be looked at by responsible individuals from the research team or from individuals from regulatory authorities.	☐
I agree to take part in this study	☐

Name of participant	Signature	Date

Name of person taking consent	Signature	Date

1 copy to participant; 1 copy to research file

MINI QUESTIONNAIRE

Dried blood spot analysis to assess adherence to cardiovascular medications

Participant Reference Number:

Q1. Have you read the participation information sheet and signed the consent form?

Y/N

Q2. Gender: M or F

Q3. Are you prescribed any cardiovascular (heart disease) medications?

Y/N

If **Yes** to Q3 please complete Table 1.

Table A2.1 Names of prescribed medicines

Approved name	Prescribed (√)	Dose (mg)	Frequency (x daily)	Approximate time since last dose (hours)
Amlodipine				
Atenolol				
Atorvastatin				
Bisoprolol				
Diltiazem				
Doxazosin				
Lisinopril				
Losartan				
Ramipril				
Simvastatin				
Valsartan				
(Not listed) *				

* Other cardiovascular medicines? Please give name.

Appendix 3 Participant information leaflet (Arabic version)

PARTICIPANT INFORMATION LEAFLET

DE MONTFORT UNIVERSITY LEICESTER

Faculty of Health and Life Sciences

Dried blood spot analysis to assess adherence to cardiovascular medications

تحليل بقعة الدم لتقييم الالتزام أدوية القلب والأوعية الدموية

ما هي الدراسة؟

هذا المشروع يعتمد على اليه قليله الايذاء للمريض لتقييم التزام المريض بادويه القلب والاوعيه الدمويه في مراكز تقديم الخدمه الاوليه والثانويه

أمراض القلب والأوعية الدموية هي واحدة من أكبر أسباب الوفاة التي تؤثر في جميع أنحاء العالم في 1 من 3 أشخاص في المملكة المتحدة. لرعاية الحالية لهؤلاء المرضى وعلى نحو متزايد للمرضى الذين اعمارهم أكثر من 50 عاما ويتضمن العلاج مزيج من الأدوية بما في ذلك حاصرات بيتا (BB)، مثبطات ACEوالادويه الخافظه للدهنيات في الدم. وهناك ادله على ان هناك ما يصل إلى 60٪ من المرضى لا يلتزمون باخذ عقاقير القلب والاوعيه الدمويه كما منصوص عليه مما يودي الى زياده نسبه الاعتلال والوفيات وارتفاع تكاليف الرعايه الطبيه . التكاليف المتوقعه لعدم استخدام الادويه في المملكه المتحده هي تقريبا 4 بليون باوند سنويا . اختبار بسيط لمراقبه تركيز الادويه في الدم سيكون ذو قيمه عاليه.

ماذا سيحدث؟

برنامج الدراسه يتضمن اختبار متقدم ومصادق عليه معتمدا على مبدا التحليل لبقعه الدم الجافه للكشف ومعرفه تراكيز الادويه المستخدمه في علاج امراض القلب والاوعيه الدمويه. وسوف تستخدم هذه الطريق التحليليه لاختبار عينات الدم والتي يتم الحصول عليها من المشاركين الذين يتناولون ادويه القلب والاوعيه الدمويه لتاكيد الاكتشاف الناجح لهذه الادويه في الدم.

كيف لك أن تشارك؟

بعد قراءه المعلومات عن البحث سيطلب منك التوقيع على استماره المشاركه قبل اعطاء وكما يطلب منك مليء استبيان صغير وستكون المعلومات في هذا الاستبيان مايلي

1. الادويه المستخدمه من قبلك
2. اخر وقت تم اخذ اخر جرعه من ادويه القلب والاوعيه الدمويه
3. الجرعه الموصوفه

جميع المعلومات عن فيما يتعلق بالعينه والاستبيان ستكون محفوظه وتمتع بالخصوصيه

كيف يتم جمع عينة بقعة الدم؟

النهج العام في هذا الاختبار يتضمن الحصول على قطره او قطرتين من الدم بواسطه اجراء بسيط يتضمن وخز الاصبع وتوخذ كميه صغيره تعادل 25 مايكرومل وسيتم وضع هذه العينه على ورقه خاصه او عن طريق تقنيات اخرى وتترك العينات لتجف في درجه حراره الغرفه لمده 2-3 ساعات على الاقل .اخذ العينات يمكن ان يكون في اي مكان على سبيل المثال في المنزل او المختبر او في عياده التمريض او في الصيدليه وبعدها يتم ارسال العينات الى المختبر.

كيف يتم تحليل بقعه الدم؟

في المختبر يتم اخراج منطقه معينه من العينه بشكل مباشر او عن طريق قص منطقه من العينه الموجوده على الورقه الخاصه .

هل ستبقى مشاركتي في البحث سريه؟

وستبقى جميع المعلومات التي يتم جمعها عنك أثناء البحث في قاعدة بيانات محمية بكلمة مرور وسرية تامة .سيتم إزالة

رمز إشارة التي سيتم استخدامها بدلا من اسمك وأية معلومات تعريفية قد تعطي.

ماذا سيحدث لنتائج الدراسة؟

وسوف تكون نتائج الدراسه جزءا أساسيا من رسالة الدكتوراه في الصيدلة السريرية في جامعة دي مونتفورت، ليستر

من يمول هذا البحث

هذا البحث هو لدراسه لدرجة الدكتوراة في جامعة دي مونتفورت , ليستر وتموله وزارة الصحة العراقية، دائره صحة ميسان.

من يتابع ويشرف على الدراسه؟

وقد استعرضت هذه الدراسة والموافقة عليها من قبل جامعة دي مونتفورت، كلية الصحة وعلوم الحياة ولجنة أخلاقيات البحث في دائره صحة ميسان.

ارشادات جمع العينه

المحتويات :

1.بطاقات جمع العينات

2. وساده كحول للتعقيم

3. لانسيت

4. شاش

5.بلاستر

6. كيس من البلاستك لحفظ العينات

1. تعبئه الرقم المرجعي للمشارك على بطاقه جمع العينات

2. تدفئه جلد الاصبع بالدلك الخفيف

3. تعقيم موقع اخذ النموذج بالكحول وتركه ليجف

4. وخز الاصبع ومسح اول قطره بالشاش المعقم

5. اضغط ضغطا خفيفا بالقرب من موقع الوخز من اجل الحصول على العينه

6.السماح لقطره الدم الاصبع للسقوط الحر على الورقه الخاصه في المنطقه المخصصه للعينه

7. تجنب لمس بطاقه اخذ العينات او نشر العينه حيث ان ذلك يودي الى جعل العينه غير صالحه

8.السماح لقطرات متعدده من السقوط على منطقه العينه حتى يتم تغطيتها بالكامل

9.عند اكمال عينه معينه يتم الانتقال الى العينه الاخرى وعلى الاقل يتم مليء دائرتين من الورقه وهذا يكون من نفس مكان الوخز.يتم رفض العينه في حاله تكون طبقات في العينه

10. يتم تزويد المشارك بالشاش والبلاستر

11.تترك العينات لتجف من 2-3 ساعات ولا يتم حفظ العينات الا بعد التاكد من جفافها وان تحفظ بعيدا عن الحراره

12.بعد جفاف العينات يتم حفظ العينات في الاكياس البلاستيكيه وتكون مهياه للنقل عن طريق البريد العادي مع استماره الموافقه على المشاركه والاستبيان

ارشادات جمع العينه بواسطه Mitra™

1.وساده كحول

2. لانسيت

3. شاش

4. بلاستر

5.كيس من البلاستك لحفظ العينات

6.مجففات الرطوبه

1. افتح مختومة التعبئة والتغليف

2. عينات تسمية مع الرقم المرجعي للمشارك

3. سحب العينات

4.تنظيف موقع العينه وتركه ليجف

5. وخز الاصبع ومسح اول قطره بالشاش

6. الضغط الخفيف قرب موضع اخذ العينه للحصول على الدم

7. تطبيق راس اخذ العينه بزاويه وكما موضح في الخطوات 3 و 4

8. انتظر حتى يصبح راس اخذ العينه اخر ثم انتظر ثانيتين اخريتين وبسرعه ارفع الراس من من الدم

9. كرر الخطوات 7و8 مع العينات المتبقيه

10.حفظ العينات المسحوبه

11. يجهز المريض بالشاش والبلاستر

12. راس اخذ العينات يتم وضعه في الكيس مع مانع الرطوبه

13. العينات الان جاهزه للتجميع والنقل عن طريق البريد مع استماره الموافقه والاستبيان

بمن يمكن الاتصال للمزيد من المعلومات؟

د. سانكيتا تانا

مدرسه ليستر للصيدله

جامعه دي مونت فورت

كيت وي

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هاتف 0116 2078274

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CONSENT FORM

استماره الموافقه

بقعه الدم الجافه لتقييم الالتزام بادويه القلب والاوعيه الدمويه

الرقم المرجعي للمشارك:

يملىء من قبل فريق البحث

اسماء الباحثين احمد العلاق د.سانكيتا تانا د.كراهام لاوسن

أؤكد أنني قد قرأت وفهمت ورقة المعلومات للدراسة المذكورة أعلاه. وقد أتيحت لي الفرصة للنظر في المعلومات، وطرح الأسئلة، وكان هذه الإجابة مرضية. ☐

وأنا أفهم أن مشاركتي طوعية وأنا حر في الانسحاب في أي وقت دون إبداء أي سبب، دون تاثر حقوقي القانونية ☐

وأنا أفهم أن البيانات التي تم جمعها خلال هذه الدراسة، يمكن النظر فيها من قبل الأفراد المسؤولين عن فريق البحث أو من الأفراد من السلطات التنظيمية. ☐

أنا أوافق على المشاركة في هذه الدراسة ☐

اسم المشارك **التوقيع** **التاريخ**

اسم الشخص الذي اخذ الموافقه **التوقيع** **التاريخ**

نسخه الى المشارك ونسخه الى الملف

MINI QUESTIONNAIRE

استبيان الالتزام

بقعه الدم الجافه لتقييم الالتزام بادويه القلب والاوعيه الدمويه

الرقم المرجعي للمشارك:

س 1. هل قراءت معلومات المشاركه بالبحث ووقعت استماره الموافقه نعم لا

س 2.الجنس ذكر او انثى

س 3. هل تصرف لك ادويه القلب والاوعيه الدمويه نعم لا

في حاله الاجابه بنعم يرجى مليء الجدول رقم 1

جدول رقم 1 اسماء الادويه الموصوفه

اسم الدواء	موصوف	الجرعه ملغم	عدد الجرعات اليوميه (يوميا)	الوقت التقريبي بالساعات لاخر جرعه
Amlodipine				
Atenolol				
Atorvastatin				
Bisoprolol				
Diltiazem				
Doxazosin				
Lisinopril				
Losartan				
Ramipril				
Simvastatin				
Valsartan				
اخرى *				

اخرى يرجى ذكر اسم الدواء